The role of beta-catenin in development of origin-specific leukaemia stem cells

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Declaration

I hereby declare that I alone composed this thesis and that work presented here is my own, except where started otherwise.

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Abstract

While we and others have prospectively identified potential origins of leukaemic stem cells (LSCs) resulting in phenotypically identical myeloid leukaemia, the functional differences among these origin-specific LSCs have not been vigorously investigated. Recently, our lab has shown that β-catenin, which is dispensable for normal HSCs, is essential for MLL LSCs, highlighting it as a potential therapeutic target for selective eradication of LSCs while sparing normal HSCs. Here, I investigated the role of β-catenin in LSCs of distinctive cellular origins using both RTTA (in vitro) and bone marrow transplantation (in vivo) assays. I demonstrated that conditional deletion of β-catenin abolished the leukaemogenic potential of LSK-Meis1-Hoxa9 pre-LSCs whereas CMP-Meis1-Hoxa9 pre-LSCs were able to develop into LSCs in vivo regardless of β-catenin status. Interestingly, conditional inactivation of β-catenin abolished the in vivo leukaemogenic property of GMP-MLL pre-LSCs whereas LSK-MLL pre-LSCs were still able to induce leukaemia in vivo in the absence of β-catenin, revealing functional differences in origin-specific LSCs in spite of their ability to induce phenotypically identical leukaemia. Since a major function of β-catenin is to mediate stem cell self-renewal, I hypothesized the presence of multiple alternative self-renewal pathways in normal HSCs that may allow the LSK/HSC-origin specific LSCs overcoming inactivation of β-catenin. Hox genes have been previously shown by others and us as key players in mediating self-renewal in normal and malignant haematopoiesis. Consistently, Hoxa9 exhibited functions as β-catenin in mediating developing of MLL pre-LSCs originated from GMP but not HSCs. To test if Hoxa9 may compensate the loss of β-catenin in LSK-MLL pre-LSCs, I have created a conditional β-catenin/Hoxa9 knockout model where a combination of these two molecules can be inactivated in normal HSCs and various myeloid progenitors. As a result, I demonstrated that specific inactivation of β-catenin or *Hoxa9* abolished the oncogenic potentials of GMP-MLL pre-LSCs, but not LSK-MLL pre-LSCs. Strikingly, inactivation of both pathways suppressed oncogenic transformation by LSK-MLL pre-LSCs and resulted in down-regulation of several Meis1-Hoxa9 target genes. Among them is *Prmt1*, which has been identified as a critical epigenetic modifying enzyme associated with an oncogenic MLL fusion complex. *Prmt1* knockdown inhibited clonogenic activity of LSK-MLL-ENL LSC with Hoxa9 or β-catenin knockout, suggesting *Prmt1* as a key modulator for transformation of LSK-MLL-ENL LSC. Together, I have demonstrated, for the first time, specific molecular and functional differences among origin-specific LSCs, in which crosstalk between multiple self-renewal pathways inherited from the cell of origins may determine the biology of the disease and mediates resistance to potential targeted therapies.

Table of contents

List of figures

List of tables

Abbreviations

ABL	Abelson murine leukemia
ALDOC	Aldolase C, fructose-bisphosphate
AF9	ALL1 fused gene from chromosome9
AGM	Aorta-gonad-mesonephro
ALL	Acute lymphoblastic leukaemia
AMBP	Alpha-1-microglobulin/bikunin precursor
AML	Acute myeloid leukaemia
APC	Adenomatous polyposis coli
APL	Acute promyelocytic leukemia
ATRA	All-trans retinoic acid
BCR	Breakpoint cluster region
BMI-1	B lymphoma Mo-MLV insertion region 1 homolog
C1QBP	Complement component 1 Q subcomponent-binding protein
CAM	Cell adhesion molecule complex
CAMP	Cathelicidin antimicrobial peptide
CBFα	core binding factor-α
CD	Cluster of differentiation
CDX	Caudal type homeobox
CDX2	Caudal type homeobox transcription factor 2
CEBPα	CCAAT/enhancer-binding protein alpha
CFC	Colony-Forming Cell
CHPF	Chondroitin polymerizing factor
CK1	Casein kinase 1
CLL	Chronic lymphocytic leukemia
CLP	Common lymphoid progenitor
CML	Chronic myeloid leukaemia
CMP	Common myeloid progenitor
Cre	Cre recombinase/ Causes recombination
CTNNB1	Catenin (cadherin-associated protein), beta 1
CXCL	Chemokine (C-X-C motif) ligand

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphates
EBF1	Early B cell factor 1
EDTA	Ethylenediaminetetraacetic acid
ENL	Eleven nineteen leukaemia
ER	Estrogen receptor
FAB	French-American-British system
FACS	Fluorescent activated cell sorting
FCS	Fetal calf serum
FDG	Fluorescein Digalactoside
FLT3	FMS-related tyrosine kinase 3
FOS	FBJ osteosarcoma oncogene
Fz	Frizzled
FZD3	Frizzled-3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS7	Growth arrest-specific protein 7
GATA1	GATA-binding factor 1
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
GSEA	Gene Set Enrichment Analysis
GSK3	Glycogen synthase kinase 3
HRP	horseradish peroxidase
HRX	Histone-lysine N-methyltransferase
HSC	Haematopoietic stem cell
IFITM1	Interferon induced transmembrane protein 1
IL3	Interleukin 3
IPCEF1	Interaction protein for cytohesin exchange factors 1
IRF4	Interferon regulatory factor 4
KRT19	Keratin 19
LATF	Leukaemia-association transcription factor

LEF	Lymphoid enhancer factor
Lox P	Llocus of X-over P1
LRP	Low density lipoprotein receptor-related protein
LSC	Leukaemia stem cell
LT-HSC	Long term-haematopoietic stem cell
LYX1	Lymphocyte antigen X 1
LYZ2	Lysozyme 2
MAF	V-maf musculoaponeurotic fibrosarcoma oncogene
MAP6	Microtubule-associated protein 6
MBOAT2	Membrane bound O-acyltransferase domain containing 2
M-CSF	Macrophage stimulating-factor
MEF2C	Myocyte enhancer factor 2C
MEP	megakaryocyte-erythroid progenitor cell
MLL	Mixed lineage leukemia
MMP	Matrix metalloproteinase
MN1	Meningioma 1
MOGAT2	Monoacylglycerol O-acyltransferase 2
MPL	Myeloproliferative leukemia virus oncogene
MPP	Multipotent progenitor cell
MSCV	Murine stem cell virus
MYC	Myelocytomatosis oncogene
NADP	Nicotinamide adenine dinucleotide phosphate
NES	Normalized enrichment score
NHR1	N-terminal hydrophobic membrane association regions
NOLC1	Nucleolar and coiled-body phosphoprtein 1
NQO1	NAD(P)H dehydrogenase, quinone 1
NUP98	Nuclear pore complex protein 98 kD
OLFM4	Olfactomedin 4
PAX5	Paired box 5
PBS	Phosphate buffer saline
PBX1	Pre-B-cell leukemia transcription factor 1
PcG	Polycomb group

PCR	Polymerase chain reaction
PI	Propidium iodide
PLA1A	phospholipase A1 member A
PLK2	Polo-like kinase 2
PML	Promyelocytic leukaemia
PRMT1	Protein arginine N-methyltransferase 1
PROS1	Protein S (alpha)
PTEN	Phosphatase and tensin homolog
PTPRF	protein tyrosine phosphatase receptor type F
PRC1	Polycomb group repressive complex 1
PRC2	Polycomb group repressive complex 2
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RARα	Retinoic acid receptor alpha
RHOD	Ras homolog family member D
RNA	Ribonucleic acid
RT	Room temperature
RTTA	Retroviral transduction/transformation assay
RUVBL2	RuvB-like AAA ATPase 2
SAMD5	Sterile alpha motif domain containing 5
SCF	Stem cell factor
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
shRNA	small hairpin RNA/short hairpin RNA
SOX9	SRY (sex determining region Y)-box 9
STC1	Stanniocalcin 1
ST-HSC	Short term-haematopoietic stem cell
TCF	T cell factor
TEL	Translocation ETS leukaemia
TGFBR3	Transforming growth factor beta receptor III
TGM2	Transglutaminase 2
THBS1	Thrombospondin 1
TIFAB	TRAF-interacting protein with forkhead-associated domain,

	family member B
TRIB1	Tribbles pseudokinase 1
TRX	Trithorax
VLDLR	Very low density lipoprotein receptor
VSV-G	Vesicular stomatitis virus glycoprotein epitope
WHO	World health organization
Wnt	Wingless type
YFP	Yellow fluorescent protein

Chapter 1 Introduction

1.1 Haematopoietic stem cells and normal haematopoiesis

Stem cells are defined as cells which possess hallmark abilities to long-term self-renew (*i.e.* to maintain stem cell pool) and differentiate into multi-lineage cell types. Stem cells can be classified into two main groups: 1. the pluripotent stem cells, such as embryonic stem cells, which are able to give rise to fully functional ectoderm, mesoderm and endoderm developmental tissues and organs; 2. Lineage-specific multipotent stem cells, among which haematopoietic stem cells (HSCs) that can differentiate into mature cells of all haematopoietic lineages such as neutrophils, eosinophils, basophils, monocytes, erythrocytes, megakaryocytes, and lymphocytes (Metcalf 2007, Copland M. 2009). In human adult bone marrow, the frequency of HSCs are estimated to be around 1 in 3×10^6 (Pettengell et al. 1994, Wang et al. 1997). Each normal HSC is capable to divide approximately 70 times during its lifespan (finite self-renewal) (Vickers et al. 2000) to maintain HSC pool as well as differentiate into functional mature blood cells. As first pointed out by Weissman's group in the late eighties, there are two subsets of HSCs, long-term reconstituting HSCs (LT-HSCs, or 'true' HSCs) and short-term reconstituting HSCs which can differentiate into multipotent progenitors (MPPs) with little or no self-renewal capacity (Weissman et al. 2001, Dalerba et al. 2007, Z 2010). In murine bone marrow, a rare population of cells with true HSC-enrich population (Spangrude et al. 1988, Okada et al. 1992, Luis et al. 2012) was identified as being negative for lineage-specific markers, and positive for high levels of stem-cell antigen 1 (Sca1), and stem cell factor receptor c-Kit, thus collectively called $Lin^-Sca1^+c\text{-}Kit^+$ (LSK) (Scheller et al.). Additional cell surface markers including $Thy1.1^{low}$, $CD34^{low/-}$, $CD38^-$, SLAM markers ($CD48^-$, and $CD150^+$), fms-related tyrosine kinase 3 (Flt3 or Flk2)$^-$ and $rhodamine123^{low}$ (Kiel et al. 2005, Yilmaz et al. 2006, Passegue et al. 2003) have also been proposed to further define LT-HSCs markers. In contrast to LT-HSCs, murine short-term HSCs have acquired $CD34^+$ and CD38+ (Randall et al. 1996) and became positive for Flt3/Flk2 (Christensen and Weissman 2001), whereas MPPs are $CD34^+$ $CD38^-$ and $Flt3^+$ (Adolfsson et al. 2001, Yang et al. 2005).

Using a combination of $Lin^{low/-}$, $Thy1.1^{low}$, $c\text{-}Kit^{high}$, and $Sca\text{-}1^+$ markers, the frequency of LT-HSC and ST-HSC in mouse bone marrow were estimated to be around 1:5000 and 1:1000, respectively (Passegue et al. 2003) (Figure 1.1). Using severe combined immunodeficient mouse (SCID)-repopulating cell assay, human HSCs were first identified as rare Lin^- $Thy1.1^+$

$CD34^+$ and $CD38^{neg/low}$ cells (Morrison et al. 1995, Doulatov et al. 2012, Bhatia et al. 1998) and more recently defined as Lin- $CD34^+CD38^-$ $CD90^+$ $CD45RA^+$ cells (Figure 1). Since the vast majority of human HSCs isolated from cord blood and bone marrow express $CD34^+$ (Bhatia et al. 1998) , CD34 has also been used as a marker to enrich in HSCs for bone marrow transplantation (Z 2010), although some reports showed that transplantation with $CD34^-$ population can also lead to a positive repopulation (Smith 2003, Haar and Ackerman 1971). These findings illustrate the fact that both the definition and the identification of HSCs should take functional assays into account instead of just immunophenotype and cell surface markers analyses (Z 2010).

Haematopoiesis is a very complex but hierarchically well-orchestrated process leading to the production and maintenance of the different blood cell types originating from HSCs (Figure 1) during normal development or after physiological stresses such as in illness, trauma, or depletion of bone marrow cells following chemotherapy or irradiation (Kondo et al. 2003). During mammalian development, primitive haematopoiesis first occurs in the yolk sac. At around day 7.5 of mouse embryonic stage (E7.5), structure named blood islands start producing myelo-erythroid progenitors (red blood cell precursors) then lymphoid precursors (Figure 1.2). After circulation occurs, blood cells are found at other haematopoietic sites. At around E10.5, HSCs can be detected in the aorta-gonad mesonephros (AGM) region then in the placenta. Definitive haematopoiesis happens in the fetal liver (E13.5), thymus, spleen and finally the bone marrow (Figure 1.2) (Orkin and Zon 2008, Costa et al. 2012, Rudolph 2010).

In adult, about 10^{13} of bone marrow cells are produced every day to maintain homeostasis as life span of mature blood cells is limited *e.g.* red blood cell and granulocyte are about 90 days and a few hours, respectively.) (Copland M. 2009). Because normal haematopoiesis is a complex process governed by many different genes, pathways and epigenetic regulation events, it is thought that HSCs with a longer lifespan are at risk of accumulating unwanted alterations or mutations, which would be harmful for the preservation of their self-renewal and/or differentiation properties. Such events nonetheless can happen, with the consequence being that some cells escape their normal regulation and dominantly expand in an uncontrolled way: functional HSCs get progressively replaced, which results in the development of various haematological malignancies (‘blood cancers’ or leukemias).

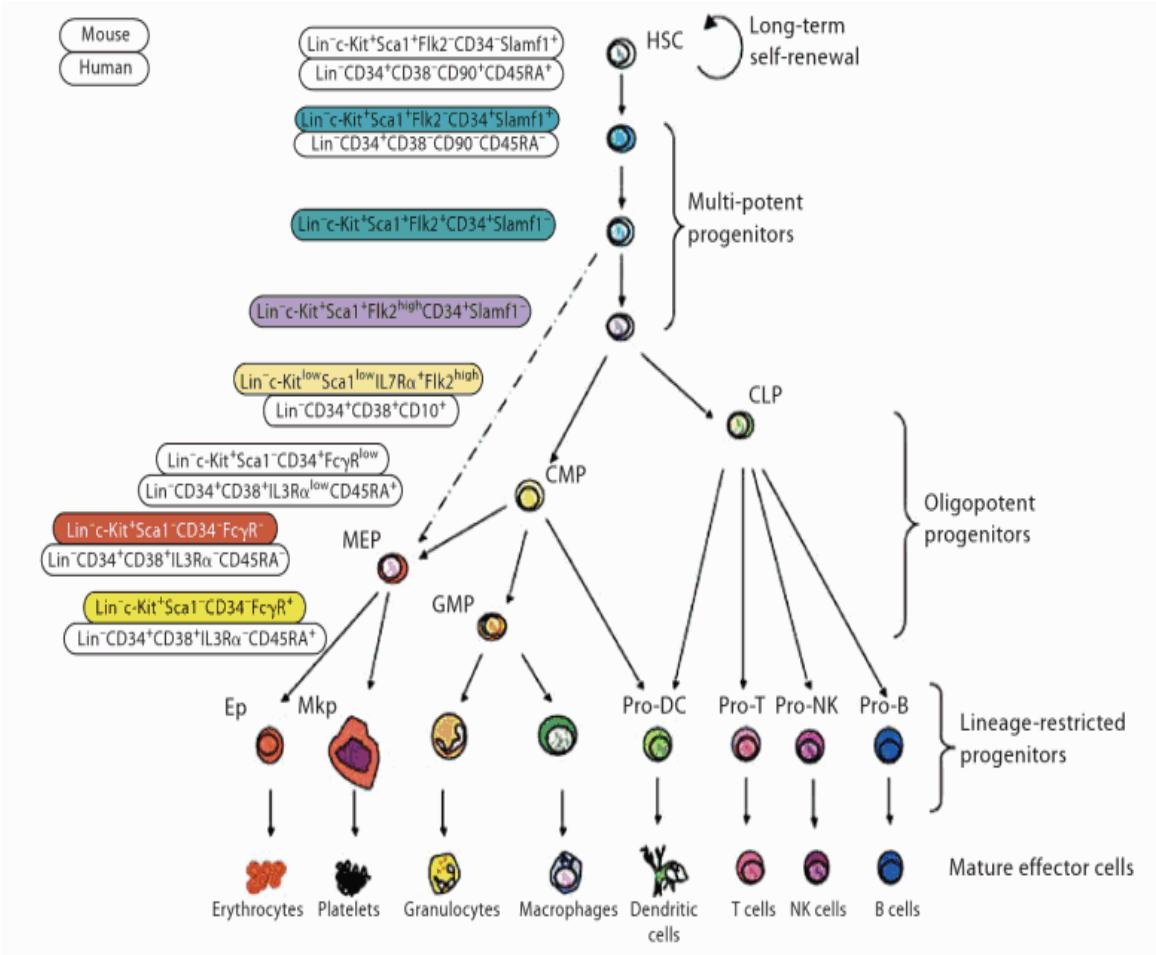


Figure 1.1 Immunophenotypic characterization of normal blood cell compartments (Rudolph 2010).

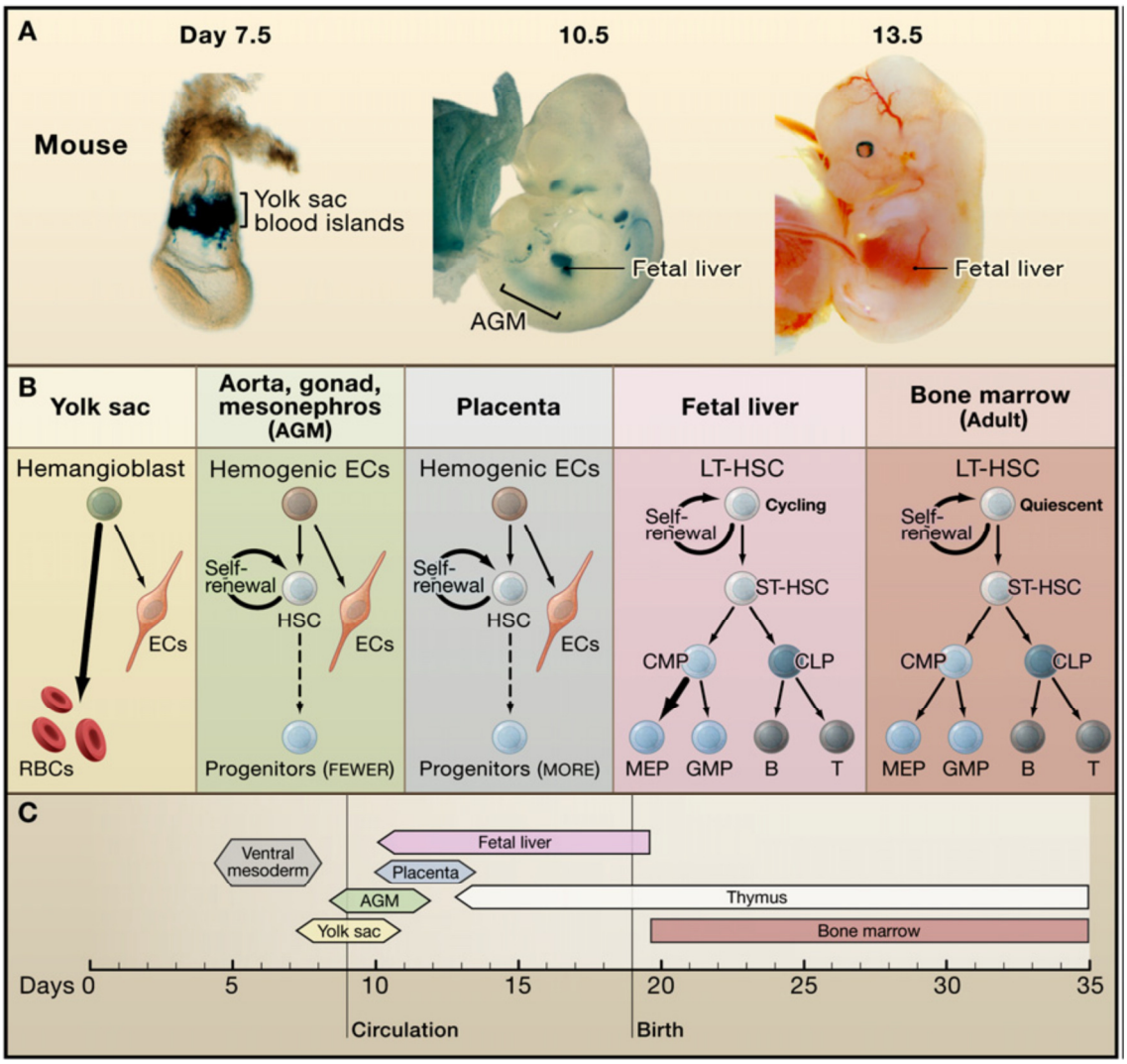


Figure 1.2: Sites of haematopoietic development in mouse. A) Primitive haematopoiesis first happens in yolk sac and later at aorta-gonad mesonephros (AGM) region, placenta, and fetal liver. B) haematopoiesis in specific location responds to produce specific blood lineages. C) Developmental time windows for shifting locations of haematopoiesis (Orkin and Zon 2008).

1.2 Acute myeloid leukaemia

Acute myeloid leukaemia (AML) is a disorder affecting haematopoietic progenitors, and is highly heterogeneous *e.g.* in cellular morphology, immunophenotype, genetic alterations, prognosis and therapeutic response (Drach 2008). In addition, genetics polymorphisms of particular locus such as NAD(P)H quinine oxidoreductase 1 (NQO1) C609T, cytochrome P450 family (CYP1A1*2B (Valine)), *Ras*, *Flt3*, and *c-Kit* (Estey and Dohner 2006) also increase a risk of predisposing to AML. From a pathological perspective, any genetic alteration of multipotent haematopoietic progenitors can possibly alter their normal growth, proliferation, and differentiation and be responsible for the disease (Mrozek et al. 2009). AML is the most frequent myeloid leukaemia in adults with a prevalence of 3.8 per 100000 individuals, rising to 17.9 per 100000 in 65+ year-old adults (Estey and Dohner 2006). Symptoms associated with the accumulation of cancer cells or blasts in AML patient bone marrow include anemia, fatigue, bleeding, and infection (Lowenberg et al. 1999). Apart from *de novo* AML, secondary AML can develop in patients with other haematologic malignancies e.g. severe congenital neutropenia, Fanconi's anemia, Bloom's syndrome, myelodysplastic syndromes. Furthermore, treatment with alkylating agents in some cancers such as Hodgkin's and non-Hodgkin's lymphoma, ovarian cancer, breast cancer, and multiple myeloma (van Leeuwen 1996) are able to induce secondary AML. In addition, therapy-related AML has been found in acute lymphoblastic leukaemia patient who received epipodophyllotoxins (topoisomerase II inhibitors) (Pui et al. 1991).

Analysis of samples for cell morphology, immunophenotype, chromosomal/molecular abnormalities that have prognosis significances is critical for the risk stratification of patients before treatment (Figure 1.3) (Grimwade et al. 2010). Two general classification systems have been proposed for AML diagnosis: the French-American-British FAB system (Table 1.1) identifies and classifies AML based on morphology and cytochemistry as well as a dominant presence of more than 30% of blasts cells in bone marrow, whereas the current WHO classification (Table 1.2) relies on cytogenetic findings and the blast threshold was decreased from 30% to 20% in bone marrow (Vardiman et al. 2002, Vardiman et al. 2009).

Table 1.1: The French-American-British (FAB) classification.

FAB subtype	Name	% of adult AML patients	Prognosis compared to average for AML
M0	Undifferentiated acute myeloblastic leukemia	5%	Worse
M1	Acute myeloblastic leukemia with minimal maturation	15%	Average
M2	Acute myeloblastic leukemia with maturation	25%	Better
M3	Acute promyelocytic leukemia (Raza-Egilmez et al.)	10%	Best
M4	Acute myelomonocytic leukemia	20%	Average
M4 eos	Acute myelomonocytic leukemia with eosinophilia	5%	Better
M5	Acute monocytic leukemia	10%	Average
M6	Acute erythroid leukemia	5%	Worse
M7	Acute megakaryoblastic leukemia	5%	Worse

Table 1.2: World Health Organization (WHO) classification of AML.

AML with certain genetic abnormalities • AML with a translocation between chromosomes 8 and 21 • AML with a translocation or inversion in chromosome 16 • AML with changes in chromosome 11q23 • APL (M3), which usually has translocation between chromosomes 15 and 17
AML with multilineage dysplasia (more than one abnormal myeloid cell type is involved)
AML related to previous chemotherapy or radiation
AML not otherwise specified (includes cases of AML that don't fall into one of the above groups; similar to the FAB classification) • Undifferentiated AML (M0) • AML with minimal maturation (M1) • AML with maturation (M2) • Acute myelomonocytic leukemia (M4) • Acute monocytic leukemia (M5) • Acute erythroid leukemia (M6) • Acute megakaryoblastic leukemia (M7) • Acute basophilic leukemia • Acute panmyelosis with fibrosis • Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)
Undifferentiated or biphenotypic acute leukaemias (leukaemias that have both lymphocytic and myeloid features). Sometimes called ALL with myeloid markers, AML with lymphoid markers, or mixed lineage leukaemias

Because AML is a heterogeneous disease, the therapeutic strategy, prognosis and treatment outcome vary in different subtypes of AMLs. In acute promyelocytic leukaemia (Raza-Egilmez et al.) with t(15;17)(q22;q21)/PML-RARA, patients are typically given all-trans retinoic acid (ATRA) and undergo a anthracycline-based protocol, whereas other subtypes of AML (*e.g.* t(8;21)(q22;q22)/RUNX1-RUNX1T1 or inv(16)(p13q22)/t(16;16)(p13;q22)/CBFB-MYH11) are commonly treated with intensive chemotherapy (cytarabine) with a favorable outcome. In contrast, treatment of AML patients with 3q abnormalities, deletion of 5 (del (5q)), monosomies of chromosome 5 and/or 7, patients with complex karyotype, or patient with MLL rearrangements (11q23) has very poor outcomes (Slovak et al. 2000). Differences in prognosis and treatment outcome of AML are significantly influenced by recurring chromosomal and/or genetic abnormalities that harbor oncogenic transcription factors (Schoch et al. 2001, Suciu et al. 2003, Haferlach et al. 2004, Breems et al. 2008). Thus, understanding the molecular regulation and pathways controlled by different leukaemia-association transcription factors (LATF) is mandatory to grasp the biology of the diseases and design effective tailored therapeutic strategies.

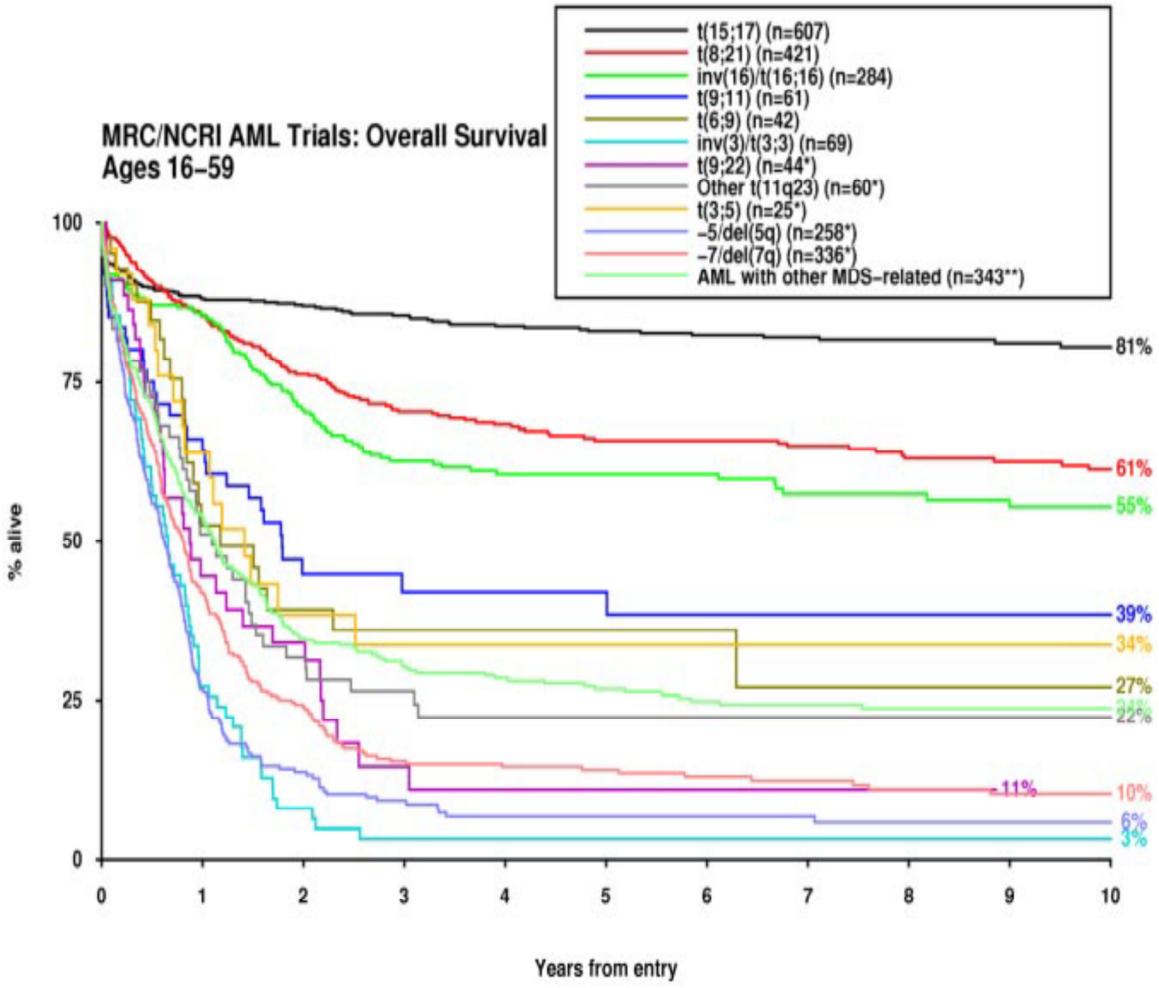


Figure 1.3: Survival curves reveal different overall survivals of AML patients as grouped based on the WHO classification system (Grimwade et al. 2010).

1.3 Transcription factors involved in acute myeloid leukaemia

Chromosomal translocations frequently observed in AML are responsible for the encoding aberrant transcriptional regulatory elements that alter the growth, self-renewal, proliferation, and differentiation of normal HSCs. About 65% of acute leukaemia patients harbor specific chromosomal translocations or abnormal chromosomal rearrangements (Figure 1.4) (Look 1997). These mostly target genes coding transcription factors that are critical for the regulation of normal haematopoiesis, and thus their deregulations play a fundamental role in the establishment and maintenance of leukaemia. The most commonly deregulated transcription factors are Homeobox (Hox) genes, MLL, AML1, PML-RARα, MN1, CBP/P300, c-Myc, ERG/TLS (Crans and Sakamoto 2001). In the present report, I will focus on some of the most important LATFs, which include members of Hox family, MLL, and MN1.

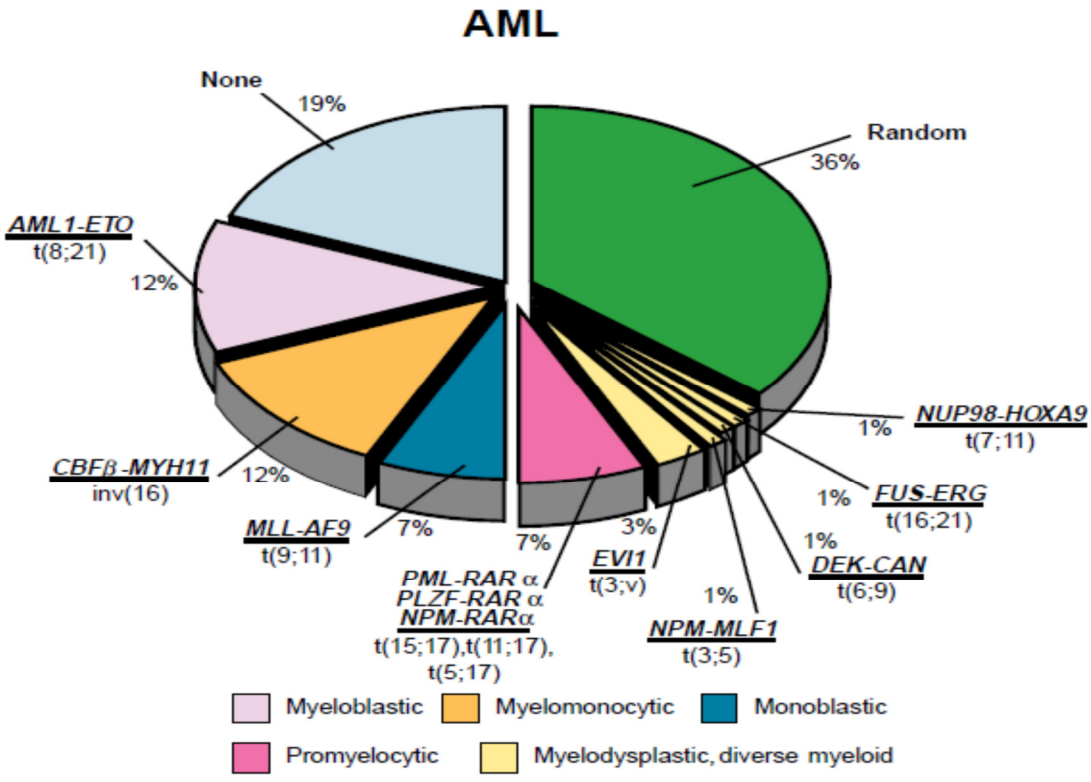
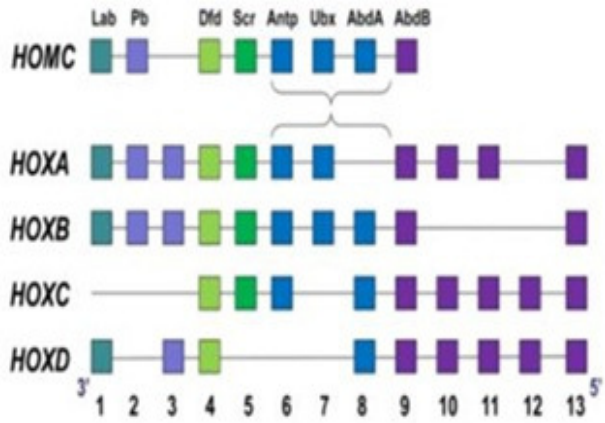


Figure 1.4: Most common chromosomal translocations and rearrangements in AML and their encoding LATF (Look 1997).

1.3.1 The Hox gene family

In human, about 39 Hox genes have been identified. In mammals, these genes are located next to each other in four clusters: HoxA to HoxD, located on four different chromosomes. Hox genes are highly conserved from *Drosophila melanogaster* to human and can be categorized into 13 groups based on their similarity of structure and function compared to Drosophila HomC cluster (Figure 1.5) (Morgan 2006, Shah N. 2010, Eklund 2011). These genes encode homeodomain transcription factors that are critical for the embryonic development as well as for mammalian definitive haematopoiesis (Eklund 2007). In the development of mammalian embryo, organ specific expression of Hox proteins have been identified including Hox1-4 dominantly expressed in the head, Hox5-7 in the thorax, and Hox8-11 in abdomen and pelvis (Eklund 2006, Eklund 2007, Eklund 2011). Hox proteins are also found essential for haematopoiesis and the maintenance of HSC pool (Frohling et al. 2007). Generally, Hox1-4 are highly expressed in $CD34^{+}$ $CD38^{-}$ population (enriched for HSC) while down regulation of those proteins is observed in more differentiated $CD34^{-/+}$ $CD38^{+}$ progenitors, which at the contrary dominantly express Hox7-11 (Sauvageau et al. 1994, Kawagoe et al. 1999).

Deregulation of Hox genes such as HoxB3, B4, A7-11 is observed in various leukaemias. In a mouse model, overexpression of HoxB3 leads to myeloproliferative disorder with accumulation of myeloid progenitors and abnormal development of early B-lymphocytes. High expression of Hoxa10 drives the expansion of megakaryocytic progenitors and reduces the number of B-lymphoid and monocytic cells, ultimately developing AML (Look 1997, Thorsteinsdottir et al. 1997). Furthermore, deregulation of Hoxa9 is commonly observed in AML (Golub et al. 1999, Lawrence et al. 1999). Kroon *et al.* (Kroon et al. 1998), showed that the co-expression of Hoxa9 and Meis1 resulted in the rapid development of leukaemia in a murine model. Although the deregulation of Hox genes, notably Hoxa9, and of their targets, is frequently observed in AML, the mechanisms that control the Hox genes expression in normal haematopoiesis and AML are not fully understood. Among the regulators of Hox genes are mixed lineage leukaemia (http://atlasgeneticsoncology.org/Genes/MLL.html) and Cdx proteins (Krivtsov et al. 2006, Schumacher and Magnuson 1997).



Chromosome and location	
Human	Mouse
7p15.2	6
17q21.3	11
12q13.3	15
2q31.1	2

Figure 1.5: Homology of mammalian Hox genes with *Drosophila melanogaster* HomC gene and chromosomal location of both human and mouse (Schumacher and Magnuson 1997).

1.3.2 Mixed lineage leukaemia

Mixed lineage leukaemia (http://atlasgeneticsoncology.org/Genes/MLL.html) gene, also known as HRX and ALL-1, is a gene homolog to *Drosophila melanogaster* trithorax (Trx) and it is located on human chromosome 11q23 (Yu et al. 1995). In drosophila, Trx protein is essential for the maintenance of cell type-specific pattern of HomC gene expression during embryogenesis (Yagi et al. 1998). In the mouse, mutations of MLL lead to severe embryo malformations such as growth retardation, haematopoietic defects, bidirectional homeotic deformation of the skeletal axis, sternum abnormalities. Moreover, MLL knockout alters the normal expression of Hox genes resulting in early death of embryos around day E10.5-E16.5 (McMahon et al. 2007, Jude et al. 2007). McMahon *et al.* (McMahon et al. 2007) showed that fetal liver of MLL knockout mice exhibited haematopoietic defects *e.g.* reduction in the number of quiescent HSCs and of both long-term and short-term HSCs. Although the conditional knockout mice had normal blood cells count, liver, spleen and thymus, MLL knockout bone marrow cells had impaired reconstitution ability in recipient mice. In addition, using a slightly different conditional murine model, Jude et al. (Jude et al. 2007) demonstrated that MLL is critical for the maintenance of adult HSCs and a deletion of MLL leads to rapid progression of bone marrow failure. Moreover, those MLL deficient cells lost their self-renewal capacity (selectively lost from mixed bone marrow chimeras) as well as their ability to intact with bone marrow environment. Together these data highlight the critical functions of MLL and Hox genes in in HSC self-renewal.

Interestingly, MLL is often translocated in AML and ALL (acute lymphoid leukaemia), and MLL rearrangement is an independent poor prognostic factor (Krivtsov and Armstrong 2007). The leukaemogeneic properties of MLL and mechanism of Hox genes deregulation have been extensively studied in MLL-translocation-bearing leukaemias (e.g. MLL fusions such as MLL-AF4 or t(4;11)(21q23), MLL-AF9 or t(9;11)(p22;q23), MLL-ENL or t(11;19)(q23;p13.3), MLL-AF10 or t(10;11)(q12;q23), and MLL-AF6 or t(6;11)(q27;q23) (Krivtsov and Armstrong 2007). In AML cases, approximately 5-10% of both children and adult patients harbor MLL rearrangements (Krivtsov and Armstrong 2007). The prevalence of different MLL fusion partners has been found to vary in adult and paediatric leukaemia (Figure 1.6). To date, there are about 73 MLL translocations and more than 50 protein partners have been cloned (http://atlasgeneticsoncology.org/Genes/MLL.html), which however share similar clinical entities and gene expression signatures (Slany 2009).

MLL is a DNA binding protein with a N-terminal DNA-binding domain and a C-terminal SET domain. In the nucleus, MLL forms a chromatin modifying complex with several other proteins. The N-terminal region is able to bind unmethylated DNA via the CxxC domain whereas the SET domain is responsible for histone methyltransferase activity (Figure 1.7) (Nakamura et al. 2002). The MLL complex is likely recruited by several transcription factors such as p53 and β-catenin (also known as Ctnnb1) to initiate transcription (Dou et al. 2005, Sierra et al. 2006). Remarkably, the transcription of Hox gene appears to be particularly dependent on MLL complex (Milne et al. 2002, Yokoyama et al. 2004). MLL rearrangements disrupt the association of the complex and therefore deregulate the transcription of its targets, especially Hox genes *e.g.* Hoxa 4, 5, 9 and 10 in pediatric MLL (Ross et al. 2004). Together, it has been suggested that the deregulation of Hox genes expression mediated by pathological MLL rearrangements leads to leukaemogenesis via uncontrolled proliferation and impairment of the differentiation of early haematopoietic stem/progenitor cells.

Of note, a cofactor of Hox called Meis1 is abundant in MLL leukaemia in parallel with Hoxa9 activation (see also section 1.3.1). Upregulation of Hoxa9 increases the number of HSCs, indicating self-renewal and co-expression of Hoxa9 and Meis1 are able to induce leukaemia in murine model (Kroon et al. 1998). In addition, Meis1-mediated apoptosis in several murine and human leukaemia cell lines is blocked by co-expressing Hoxa9 (Wermuth and Buchberg 2005).

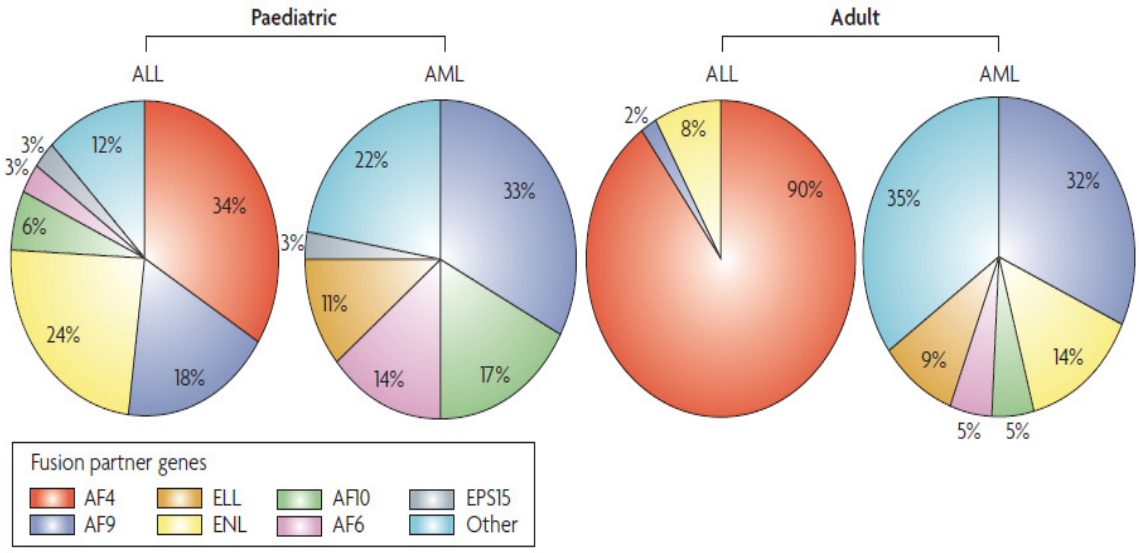


Figure 1.6: Relative frequencies of MLL rearrangements in childhood and adult leukaemias (Krivtsov and Armstrong 2007).

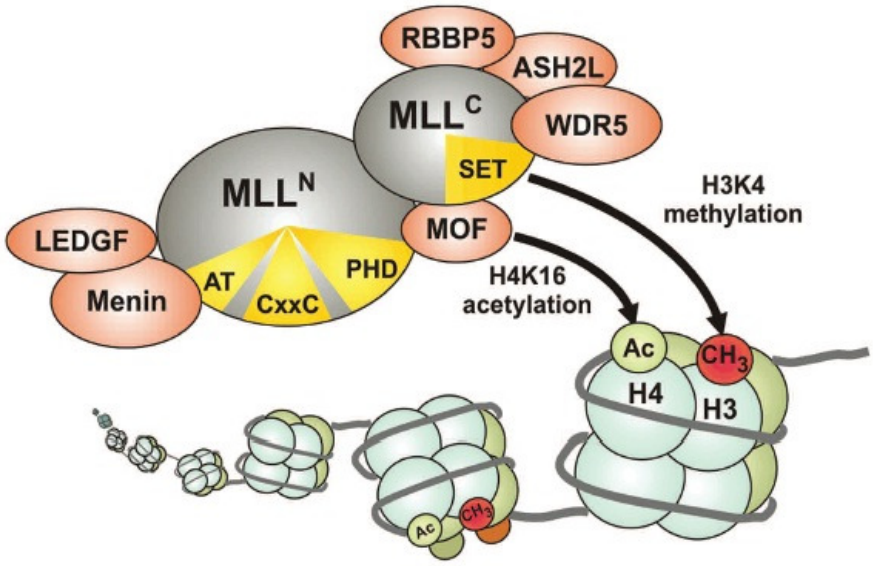


Figure 1.7: Partners of the MLL complex (Slany 2009).

1.3.3 AML1-ETO

Chromosomal translocations t(8;21)(q22;q22) resulting in AML1-ETO fusion is the most frequent one in AML (Z 2010). It accounts for up to 40% of the AML-M2 subtype in the FAB classification, with granulocytic differentiated leukemic blasts being observed (Elagib and Goldfarb 2007).

AML1 (also known as RUNX1 or CBFα2) is located on chromosome 21 and critical for early haematopoiesis (Sroczynska et al. 2009). AML1 transcription factor can bind several target sequences of genes involved in tissue-specific expression of haematopoietic genes like granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage stimulating-factor (M-CSF) and interleukin 3 (IL-3) (Crans and Sakamoto 2001). Its N-terminus (RUNXT domain) is a close homolog to the drosophila pair-rule protein runt and is essential for heterodimerization with CBFβ to bind specific DNA target sequences (Daga et al. 1992). While CBFβ alone is not able to directly bind the target sequence (Wang et al. 1993, Ogawa et al. 1993), its dimerization with AML1 is critical for the regulation of downstream target gene expression.

Fusion partner of AML1, ETO, is a homolog of *Drosophila melanogaster* Nervy and is located on human chromosome 8q22 (Crans and Sakamoto 2001). ETO possessed homodimerization and heterodimerization abilities and could form a complex that functions as a transcriptional regulator (Downing 1999). There is further evidence to suggest that ETO functions as a transcriptional regulator by corporation with nuclear co-repressors N-CoR and Sin3A to mediate histone deacetylation (Wang et al. 1998, Lutterbach et al. 1998). ETO Nervy region 2 (NHR2) invariably found in AML1-ETO fusion has a role in suppressing the transcriptional activity of AML1, which results in the blockage of myeloid cells differentiation (Liu et al. 2006).

In human AML with t(8;21)(q22;q22), the most common aberrant transcript is known as full-length AML1-ETO (AE; AML1 exons 1-5 fused to ETO exons 2-11) that contains 752 amino acids with N-terminal of AML1 (RUNX1), DNA binding domain, and ETO protein (Moroi and Sato 1975). AML1-ETO fusion protein is able to induce stem cell self-renewal and disrupt normal haematopoiesis, however AML1-ETO expression alone is not sufficient to induce leukaemi (Yuan et al. 2001, Roudaia et al. 2009). As a consequence, secondary mutations are required for AML1-ETO leukaemia (de Guzman et al. 2002). In addition to the full-length AE, Yan et al. (Yan et al. 2006), identified a variant transcript of AML1-ETO known as AML1-ETO 9a (AE9a) which contains additionally the exon 9a of ETO gene. This variant has the ability to

rapidly induce leukaemia in a mouse model. Moreover, co-expression of AE and AE9a synergistically blocks myeloid cells in a more immature stage than using AE9a alone, and has an early disease onset.

1.3.4 PML-RARα

Acute promyelocytic leukaemia is characterized by the t(15;17)(q22;q21) reciprocal translocation between human chromosomes 15 and 17 that results in the expression of two chimeric proteins called PML/RARα and RARα/PML (Grimwade et al. 1997).

RARα gene located on chromosome 17q21 encodes a retinoic acid receptor and is a member of the nuclear hormone receptor superfamily. This family of receptors is involved in normal differentiation and growth inhibition of several cell types (Leid et al. 1992, Mangelsdorf et al. 1995, Chambon 1995). Its fusion partner, PML, maps to chromosome 15q22 and belongs to the RING finger protein family. PML regulates several biological functions ranging from regulation of interferon function and immunosurveillance to pro-apoptotic and tumor suppressor functions. In addition, PML/RARα with co-transcriptional repression property results in blocking the accession of transcription factors to specific target sequence. APL has a dominant leukaemic phenotype comparable to committed myeloid progenitors (promyelocytic cells) that do not possess self-renewal capacity in a normal (*i.e.* non leukaemic) situation. Consequently, suppression of target genes including genes that function in normal differentiation of myeloid cells leads to the development of APL (leukaemic cells are arrested at the promyelocytic stage) (Testa et al. 1998, Zhong et al. 2000). APL is a subtype of AML that shows defects in the regulation of transcriptional factors involved in HSC differentiation and self-renewal processes. Current therapeutic treatment of APL with PML/RARα is all-trans retinoic acid (ATRA)-induced transcription activation of PML/RARα downstream targets and differentiation of APL cells (So et al. 2000).

1.3.5 MN1

MN1 is an evolutionarily conserved gene located on chromosome 22 that was first identified as a fusion target of t(4;22) in meningioma (Grosveld 2007). MN1 interacts with vitamin D receptor resulting in suppression of the proliferation of osteoblast cell line (Sutton et al. 2005). Similarly, growth inhibition of epithelial cells could be induced by MN1 expression (Kang et al. 2003). These suggest potential functions of MN1 in regulating growth and proliferation.

In AML, MN1 fuses with TEL in t(12;22)(p13;q12) (Grosveld 2007). MN1 is also found overexpressed in patients with inv(16) (Ross et al. 2004), patients with expressing EVI1 (Valk et al. 2004), and in adult AML with normal karyotype (Heuser et al. 2006). High expression of MN1 correlates with poor outcome and ATRA resistance in AML (Heuser et al. 2007, Meester-Smoor et al. 2008). Quantitative RQ-PCR analysis revealed that MN1 is expressed at low level in mouse HSC but not in CMP, CLP, and MEP, whereas high expression of MN1 can be observed in GMP population, suggesting MN1 could be a regulator of GMP-derived myelopoiesis (Carella et al. 2007). Recently Heuser et al. (Heuser et al. 2011) however showed that CMP but not GMP could be a target for MN1 induced leukaemia, which requires the formation of a complex with MEIS1-AbdB-like Hox protein for its leukaemogeneic properties.

1.4 Leukaemic stem cells (LSCs)

Leukaemia is the first human malignancy where cancer stem cells or leukaemic stem cells (LSCs) have been functionally isolated. LSCs are defined as leukaemic cells with infinite self-renewal capacity that sustain the growth of malignant tumors and recapitulate the disease upon transplantation (Bonnet 2005). In 1997, using a xenograft quantitative assay to identify and characterize stem cells *in vivo*, Bonnet and Dick (Bonnet and Dick 1997) made the pioneer observation that SCID leukaemia initiating cells (SL-IC) have self-renewal capacity whilst retaining the potential to differentiate and that these cells dominantly expressed CD34+ but not CD38 (similarly to normal HSCs) in all tested AML. This finding showed that, like normal haematopoiesis, AML is organized as a hierarchy and suggested that LSCs may derive from HSCs (in opposition to derive from committed progenitor cells). However, like for normal HSCs, functional biological assays are the gold standard to characterize LSCs rather than just relying on morphology and immunophenotype. Although $CD34^+$ $CD38^-$ cell were identified as human LSCs, recent report by Bonnet group (Taussig et al. 2010) demonstrate that about one-half of LSCs are restricted into $CD34^-$ fraction, whereas $CD34^+$ fraction contained normal multilineage hematopoietic repopulating cells. Thus, characterization of human LSC based on immunophenotyping may not be trivial and LSC therapies targeting against surface antigens can be challenging.

1.4.1 The origin of leukaemic stem cells

It is unclear from which cells do the LSCs originate from the normal HSCs hierarchical system e.g., HSCs and/or from more committed progenitor populations. Retroviral transduction/transformation assay (RTTA) involving the transduction of specific LATF into sorted distinct haematopoietic population is a powerful tool to identify possible targets of oncogenes and also decipher the origin of LSCs. So et al. (So et al. 2003) demonstrated that MLL-GAS7 could induce multi-lineage leukaemia in multipotent progenitor (MPP) but not in common myeloid progenitor (CMP) or common lymphoid progenitor (CLP) derived cells. On the other hand, a different fusion, MLL-ENL, was able to induce AML in HSC, CMP, and granulocyte/monocyte committed progenitor (GMP) (Cozzio et al. 2003). Interestingly, Heuser *et al.*, (Heuser M. 2011), reported that CMP but not GMP was a target for MN1-induced leukaemia and that the transformation capacity of MN1 required the MEIS1/Abdb-like Hox protein complex. Consistently, overexpression of MEIS1 and Hoxa9 or Hoxa10 was able to render GMPs susceptible to MN1-induce leukaemia, suggesting different functional requirement in LSCs derived from different cellular origins.

APL with PML-RARα is thought to originate from committed progenitors rather than HSCs: Turhan et al., (Turhan et al. 1995) showed by RT-PCR that only $CD34^+CD38^+$ (committed progenitors) but not $CD34^+CD38^-$ (HSCs) cells express PML-RARα transcripts. These findings suggested that possibly HSC are not the origin of LSCs in APL. However, fluorescent in situ hybridization (Jude et al.) evidence indicated that some APLs actually express PML-RARAα in $CD34^+CD38^-$ cells. This would suggest that the transcription activity of PML-RARα is inhibited in $CD34^+CD38^-$ transformed cells and also that a fraction of APL is sustained by cells derived from HSCs.

1.4.2 Clinical significance of the molecular pathways that are critical for the development of leukaemic stem cells

The recent development of the stem cell biology field has provided new insights into cancer biology and discloses some of mechanisms involved in the generation of cancer stem cells. It is clear that self-renewal capacity is a hallmark of normal stem cells as well as cancer stem cells. Several pathways such as the Hedgehog, Hox, polycomb/Mll, PTEN, telomerase, GSK3 and Wnt/β-catenin pathways involved in self-renewal of HSCs have also been implied in

LSCs (Copland M. 2009). In the following sections, I will discuss Hox, BMI1, GSK3 and the Wnt/β-catenin pathway.

1.4.2.1 Hox gene family in the development of LSC

As described in previous section (1.3.1 The Homeobox gene family), Hox proteins, highly expressed in primitive self-renewing HSCs and down-regulated in more differentiated progenitors, are important for normal haematopoiesis, self-renewal as well as leukaemogenesis. In mouse models, over-expression of Hoxa9 but not Hoxb4 alone was able to induce leukaemia (Huntly and Gilliland 2005). In human AML, chromosomal translocation between Nup98 and Hoxa9 or HoxD13 has been observed, and overexpression of Hoxa9 is of poor prognosis (Golub et al. 1999, Anderson and Slotkin 1975, Raza-Egilmez et al. 1998). Furthermore, expression of several Hox transcripts (*e.g.* Hoxb3, Hoxb8, and Hoxa9) has been associated with leukaemogenesis (Thorsteinsdottir et al. 1997, Perkins et al. 1990, Sauvageau et al. 1997). The deregulation of Hox gene expression can be mediated by the upstream Hox-regulators such as MLL and CDX proteins (Mlodzik and Gehring 1987). Chromosomal translocation between CDX2 and TEL, t(12;13) has also been reported in AML and it is believed that alteration of CDX2 by TEL might be a cause of leukaemogenesis (Chase et al. 1999).

1.4.2.2 BMI1

There are two polycomb group (PcG) repressive complexes, polycomb repressive complex 1 (PRC1) and 2 (PRC2). BMI1 belongs to PRC1 and is a critical molecule for the maintenance of several somatic stem cells self-renewal including HSCs (Lessard and Sauvageau 2003, Park et al. 2003, Iwama et al. 2004, Mills 2010). BMI1 also plays a role in the maintenance of multipotent property of both HSCs and MPPs via a suppression of particular lineage gene promoters (e.g., *Ebf1* and *Pax5* of B-cell lineage developmental regulator genes). Loss of Bmi1 promotes expression of Ebf1 and Pax5 in HSCs/MPPs, leading to enhanced lymphopoiesis and a marked reduction in HSC/MPPs (Oguro et al. 2010). In addition, BMI1 regulates cell senescence by targeting Ink4a/Arf locus that encodes a cyclin kinase inhibitor $p16^{Ink4a}$ and a tumor suppressor $p19^{Arf}$. Deletion of both $p16^{Ink4a}$ and $p19^{Arf}$ in BMI deficient mice leads to *de novo* impaired self-renewal potential of HSCs (Oguro et al. 2006). In a murine model, Meis1-Hoxa9 induced LSC isolated from fetal liver of BMI1 deficient mice lost their ability to long-term engraft in secondary recipient mice and progressed to terminal differentiation and apoptosis, indicating its critical function in LSC maintenance (Lessard and Sauvageau 2003).

Our lab and others have further addressed the role of Bmi1 in LSC induced by LATFs by revealing a novel functional crosstalk between BMI1 and MLL/Hoxa9 in leukaemia development. We showed that Hox-associated MLL-AF9 was able to transform BMI1 knockout cells, whereas AML1-ETO and PML-RARα that failed to activate Hoxa9 cannot transformed BMI1 knockout cells. The leukaemogeneic property of MLL-AF9 was impaired by suppression of both BMI1 and Hoxa9. Additionally, Bmi1 deficient cells transduced with AML1-ETO could overcome senescence via the ectopic activation of Hoxa9, which suppresses $p16^{ink4a}$/$p19^{ARF}$, targets of BMI1 (Smith et al. 2011). The other study Yuan et al. (Yuan et al. 2011), reported that BMI deficient GMP cells transformed with MLL-AF9 were immortalized *in vitro* with a decrease number of LSCs that induced leukaemia with a more differentiated phenotype. Co-suppression of p16 and p19 in BMI1 knockout cells could partially rescue the leukaemogeneic potential of BMI knockout GMP-MLL-AF9 cells in vivo. Together, these studies reveal a critical function of BMI1 in LSC self-renewal, and suggest that the combined targeting of BMI1 and Hoxa9 could be a way to eradicate LSCs.

1.4.2.3 GSK3 and its inhibitors

The conserved glycogen synthase kinase 3 family (GSK3) including GSK3α, GSK3β, and GSK3β2 is involved in several signaling pathways *e.g.* Hedgehog and Wnt/beta-catenin pathways to modulate cell division, stem cell self-renewal, differentiation. GSK3 is a serine/threonine kinase that is a key mediator in many human diseases. While inactivating mutations of GSK3 has been observed in several types of cancers (Cohen and Goedert 2004), GSK3 can function as a tumor promoter to support proliferation in some malignancies (Chen et al. 2009). Targeting of GSK3 may be a therapeutic option for GSK3-dependent cancers such as MLL leukaemia (Wang et al. 2008). GSK3 was able to maintain MLL LSC by promoting conditional association of CREB and its coactivator TORC and CBP with a homeodomain protein MEIS1 [(Wang et al. 2010)]. Since Hoxa9 and its co-factor MEIS1 play critical roles in MLL-induced leukaemia and other subtypes of AML, GSK3 inhibition can be a therapeutic strategy to target broad spectrum of AML by impairing the HoxA9/Meis1-induced proliferation and self-renewal of LSCs.

Recently, our group reported that MLL LSCs that acquired resistance to GSK3 inhibitors could be resensitized by the suppression of β-catenin expression (Yeung et al. 2010). These findings make the connection between MLL-induced leukaemia, GSK3 resistance, and Wnt/β-catenin pathway in the development of drug resistant MLL LSC. However, it is less clear

whether targeting of LSC with GSK3 alone or combined with the suppression of critical molecules involving in LSC self-renewal such as β-catenin could be an efficient therapeutic option to eradicate other type of LSCs.

1.5 Wnt/β-catenin involvement in LSC development and self-renewal

β-catenin is an adherence junction protein coded by the Ctnnb1 gene located on human chromosome 3 (p22.1) playing a crucial role in the development and maintenance of cell epithelial layers. The protein was first identified in association with the cell adhesion molecule complex (Mulloy et al.) E-cadherin (in the E-Cadherin-catenin complex). β-catenin functions as a linker between E-cadherin and α-catenin to the actin cytoskeleton protein in cell-to-cell adhesion process (Brault et al. 2001). In addition, McCrea et al. (McCrea et al. 1991), and Butz et al. (Butz et al. 1992), demonstrated that the Drosophila Armadillo homologue β-catenin is an important part of Wnt signalling pathway (Brault et al. 2001, Barker and Clevers 2000). Recent studies demonstrated that Wnt signaling pathway is critical for control cell proliferation, differentiation, cell polarity, and cell fate during the development of embryo as well as adult homeostasis. Unsurprisingly, genetic alterations of the Wnt pathway lead to several birth defects, cancers and other diseases (Clevers 2006).

There are three different Wnt signaling pathways: the canonical Wnt/β-catenin pathway, the non-canonical planer cell polarity pathway, and the Wnt/Ca^{2+} pathway (Clevers 2006). Here, I will focus on the Wnt/β-catenin canonical cascade as there is strong evidence for its involvement in the control of normal and malignant haematopoiesis.

In the absence of Wnt, cytoplasmic β-catenin is part of the destruction complex with Axin protein, tumor suppressor adenomatous polyposis coli (APC), GSK3, and casein kinase 1 (CK1). GSK3 and CK1 consecutively phosphorylate β-catenin at its N-terminus targeting it for proteosomal degradation after the recognition of E3 ubiquitin ligase β-Trcp. As a consequence, T cell factor/lymphoid enhancer factor (TCF-LEF)/Groucho interacts with histone deacetylase (HDAC) and Wnt target genes are not activated (Figure 1.8 A). In the presence of Wnt ligand, the co-receptor complex is formed between Frizzled (Fz) and low-density lipoprotein receptor-related protein 6 (LRP6) or close family member LRP5, which leads to the recruitment of Dishevelled (Dvl). This allows the phosphorylation and activation of LRP5/6 after Axin recruitment to the co-receptor complex. This process means that the destruction complex is

dismantled and thus the Axin-mediated β-catenin phosphorylation/degradation is blocked, leading to an accumulation of β-catenin in the nucleus where it gets active and functions as a co-activator for TCF to activate Wnt target genes such as c-Myc, n-Myc, cyclin D, Tcf-1, Sox9, and CD44 (Figure 1.8 B) (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/ , MacDonald et al. 2009).

Since Wnt/β-catenin canonical pathway is very important in homeostasis and regulation of cell proliferation, differentiation and cell fate decision, cell development, mutations of its components are commonly found in many diseases such as hereditary defects and cancers (MacDonald et al. 2009). Mutations of tumor suppressors APC and Axin have been reported in many tumors including Familiar Adenomatous Polyposis tumor (FAP) (Nishisho et al. 1991), sporadic colorectal cancers (Kinzler and Vogelstein 1996). Abraham et al. (Abraham et al. 2001), demonstrated that 75% of sporadic juvenile nasopharyngeal angiofibromas carried beta catnin mutations. Moreover, Moreno-Brueno et al. (Moreno-Bueno et al. 2001), reported that high expression of β-catenin have been found in all 40 of the pilomatricomas they studied, and further investigation revealed that 3 of 11 (26%) samples carried mutations on the GSK3β site responsible for its phosphorylation activity. β-catenin mutations are also found in reproductive organs tumors including the endometrial and prostate cancers (Voeller et al. 1998, Ashihara et al. 2002). An example of activating mutations on β-catenin lies in hepatocellular carcinoma associated with hepatitis C (HCC) (Huang et al. 1999, Legoix et al. 1999, Wong et al. 2001). Moreover, the study of Satoh et al. (Satoh et al. 2000), revealed that about 14 % of HCCs harbored activating mutations on the phosphorylation sites of beta catenin and about 9 % carried inactivating mutation of tumor suppressor, Axin1 gene. Thus, based on many those literatures, alterations in Wnt/β-catenin pathways are important for the etiology of several solid tumors.

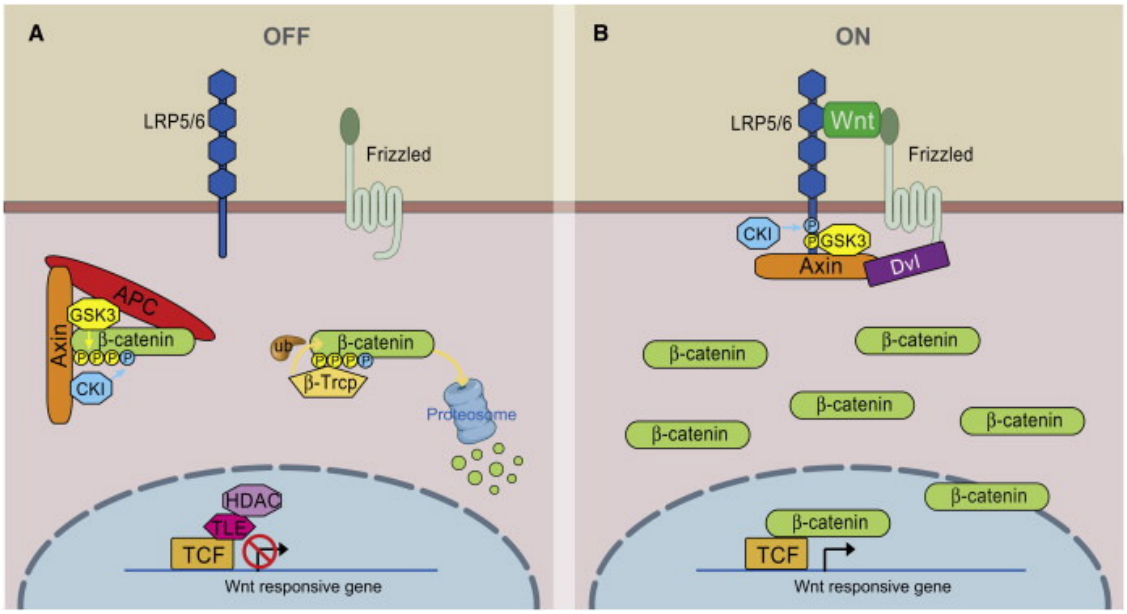


Figure 1.8: Wnt/β-catenin pathway in the absence (A) or in the presence of Wnt ligand (B) (MacDonald et al. 2009).

In the present report, I will focus on the role of Wnt/β-catenin in self-renewal its potential as a therapeutic target for haematopoietic malignancies. Involvement of β-catenin in the control of normal haematopoiesis and its pathogenesis emerged in the report by Reya et al. (Reya et al. 2000) showing that proliferation of B lymphocyte progenitors were significantly controlled by Wnt/β-catenin signaling pathway. They subsequently further investigated the impact of β-catenin on HSC regulation, and found that transduced HSCs with activated β-catenin could retain cells in an immature state which express c-kit^{+} Thy1.1^{lo} Lin^{-} Sca-1^{+} (see phenotype of HSCs in section 1.1). In addition, they demonstrated that all recipient mice transplanted with activated β-catenin-transduced HSCs cells could engraft and reconstitute the haematopoietic system (myeloid and lymphoid lineages) with a high level of chimerism. These results revealed a role for active β-catenin in blocking differentiation whilst promoting HSC proliferation (Reya et al. 2003). A few years later, Scheller et al. (Scheller et al. 2006), using Cre-LoxP technology to modulate the activation of β-catenin in mice, elegantly demonstrated an important function of β-catenin in the control of normal haematopoiesis. They found that constitutive activation of β-catenin lead to the death of the recipient mice because of HSCs fail to repopulate the haematopoietic system and an arrest in multilineage differentiation. Furthermore, Kirstetter et al. (Kirstetter P 2006), demonstrated that activation of Wnt signalling for HSCs by conditional

expression of a stable form of β-catenin (β-catS33Y) leads to block differentiation and maturation of multilineage myeloid progenitors which are caused by the alteration of several critical cell signalling molecules including Cdkn1a, sfpi1, Hoxb4, Bmi1, and integrin. These results suggest that super-physiological level of β-catenin may have inhibitory effects on normal HSC functions.

In contrast to the study of the gain of function of β-catenin in normal haematopoiesis, Cobas et al. (Cobas M 2004), demonstrated that inducible Cre-loxP-mediated deletion of β-catenin (MxCre) in bone marrow cells could not affect self-renew capacity of β-catenin deficient bone marrow cells. β-catenin deficient cells able to reconstitute and give rise to all full functional hematopoietic lineages (myeloid, erythroid, and lymphoid). Moreover, Jeannet et al. (Jeannet G 2008) reported that HSCs with combined genetic deletion of β-catenin and γ-catenin are able to maintain their long-term repopulation capacity as well as multilineage differentiation of both myeloid and lymphoid lineages. Interestingly, subsequent experiments by this group revealed that T-cell development via TCF-1 activation is independent of a functional interaction of β-catenin and γ-catenin (reference). In addition, Prlic and Bevan (Prlic M 2011), demonstrated that conditionally deletion of β-catenin in mature T cells did not affect memory T cell functions, in which migration and response to chronic infection or lymphopenia induced proliferation. Apart from genetic modification approaches, the investigation of role of β-catenin in normal haematopoiesis by ectopic expression of Wnt/β-catenin inhibitor using GSK3β (Chase et al.) also did not resulting in the alteration of HSC differentiation (Moon RT 2004). These results indicated that β-catenin is dispensable for both hematopoiesis and lymphopoiesis.

Alongside its major function in normal haematopoiesis, there have been many reports evaluating the role of Wnt/β-catenin pathway in leukemogenesis. In chronic myeloid leukemia (CML), loss of β-catenin results in a self-renewal defect of CML LSCs (Zhao et al. 2007). In the absence of β-catenin, the progression of CML mediated by BCR-ABL fusion gene slowed down, whereas acute lymphoblastic leukemia (ALL) development was unaffected. This clearly showed that different requirements of β-catenin exist in different types of leukaemia induced by BCR-ABL. Lu et al. (Lu et al. 2004), observed that important Wnt ligands including Wnt3, Wnt5b, Wnt6, Wnt10a, Wnt14, and Wnt16 as well as Fzd3 receptor are overexpressed and activated in B cell chronic lymphocytic leukemia (CLL) compared to normal B cells. Targeting of GSK3 by SB-216763 helped promoting CLL survival, whereas the nonsteroidal anti-inflammatory drug R-Etodolac induced CLL cells apoptosis. For the role of β-catenin in normal HSC, Zhao et al.

reported a loss of long-term stem cell maintenance upon serial transplant although β-catenin deficient mice were able to have seemingly functional HSCs. On the other hand, other studies showed that bone marrow cells derived from mice deficient in β-catenin or even in combination with γ-catenin did not have any obvious haematopoietic defects under both normal and stress conditions and are capable of fully reconstituting hematopoietic systems upon bone marrow trans-plantation into syngeneic mice (Cobas et al. 2004, Jeannet et al. 2008, Koch et al. 2008). These results suggest a largely dispensable function of β-catenin in normal HSCs with the potential exception of under special extremely conditions.

In AML, Muller-Tidow et al. (Muller-Tidow et al. 2004), further highlighted the importance of the Wnt/beta-catenin pathway using a high-density oligonucleotide arrays to identify candidate genes in U937 human leukaemic cell line, after transfection with three different LATFs *i.e.* AML1-ETO, PML-RARα, PLZF-RARα. They found that among the genes controlled by those LATFs (38 down-regulated and 14 up-regulated) many are components of the Wnt/β-catenin pathway like γ-catenin and β-catenin as well as TCF /LEF, cyclin D1 and c-Myc.

Wang et al., 2010 (Wang Y 2010), studied the impact of β-catenin on self-renewal property and the development of LSCs by transducing either HSCs (purified as Lin^- c-Kit^+ Sca-1^+) or more differentiated progenitors -GMPs (purified as Lin^- c-Kit^+ Sca-1^- $FcR\gamma^+$ $CD34^+$) with MLL-AF9 fusion or Meis1 and Hoxa9 oncogenes. Strikingly, there was no significant difference between HSCs and GMPs when co-transduced with Meis1 and Hoxa9 oncogenes or MLL-AF9 in terms of proliferation, cell cycle analysis, apoptotic rate, immunophenotype or the cell morphology during six week in culture. However, after transplantation of the transformed cells into recipient mice, 93% of mice transplanted with MLL-AF9 GMPs and 70% of mice transplanted with Meis1-Hoxa9 HSCs developed AML. In contrast, only 4% (1 out of 23 mice) with Meis1-Hoxa9 GMPs developed AML. Active β-catenin was expressed in Meis1-Hoxa9 HSCs and MLL-AF9 GMPs but not in normal GMPs and the Meis1-Hoxa9 GMPs derived cells, suggesting that active β-catenin was required for the Meis1-Hoxa9 GMPs to develop the disease. Consistently transduction of Meis1-Hoxa9 GMPs with the active β-catenin resurrected its transformation ability and induced leukemia in mice similar to those by Meis1-Hoxa9 LSKs. In contrast, knockout β-catenin in Meis1-Hoxa9 HSCs or MLL-AF9 GMPs compromised their leukaemogenic potentials in mice.

On the other hand, our group (Yeung et al. 2010) demonstrated that β-catenin was activated during the development of MLL LSCs. Using global expression analysis of the pre-leukemic-like state MLL-ENL transformed cells (pre-LSC) and the MLL-ENL LSC enriched population, we found that several Wnt/β-catenin components including Frizzled 4/6 and Wnt targets such as cyclin D2 were upregulated in the MLL-LSCs compared to MLL-pre-LSC. shRNA mediated down-regulation of β-catenin reverted the MLL LSCs to a pre-LSC like state which were sensitive to GSK3 inhibitor (GSK3i) treatment, suggesting that β-catenin might functions in the establishment of drug resistance in MLL leukemia. Also, the validity of the β-catenin silencing approach was also confirmed in human leukemic cell lines and primary samples (Yeung et al. 2010, Gandillet et al. 2011). In addition, complete knockout of β-catenin in LSC abolished oncogenic property after transplantation to mice. Hence, β-catenin is required for the development of MLL-ENL LSCs. Since β-catenin is largely dispensable for normal HSC functions, these findings highlight the therapeutic potentials of targeting β-catenin in MLL LSCs. However, further cellular and molecular dissection of the functions of Wnt/β-catenin pathway involved in various LSCs of distinctive cellular origins are absolutely fundamental to understand the pathology of the disease and design effective therapeutics strategies.

Chapter 2 Objectives

Human acute leukaemia is characterized by the presence of specific and recurring genetic mutations that frequently result in deregulation of transcriptional program of the targeted cells. Emerging evidence indicate that the biology, clinical behaviour and therapeutic responses of leukemic cells are largely dependent on the initiating genetic events. Thus, characterization of the molecular functions and the origins of these genetic mutations are essential for better understanding the biology of the disease and the development of specific inhibitors for cancer therapy**.** Recently, our group demonstrated the critical function of β-catenin in the development of MLL LSCs and its function in the establishment of drug resistant MLL LSCs derived from c-Kit positive cells. However, it is still largely unknown whether β-catenin also plays the same role in the development and maintenance of LSCs derived from different HSC/progenitors induced by common LATFs. In my PhD project, I investigated the impact of β-catenin in the initiation and maintenance of LSCs induced by some of the most common LATFs including MLL-AF9, MLL-ENL, MN1, Meis1-Hoxa9, and E2A-PBX1. I also studied the requirement of β-catenin in LSCs derived from different haematopoietic populations.

The specific aims of my PhD project are followings:

1) To explore the origin of leukemic stem cells initiated from certain types of LATFs and their β-catenin dependency
2) To identify downstream targets of β-catenin in different LSCs and explore potential therapeutic targets in different LSCs
3) To study a potential functional crosstalk between β-catenin and Hoxa9 in MLL LSCs

Chapter 3 Materials and methods

3.1 Mice

6-7 week-old C57BL/6 (CD45.2) or SJL (CD45.1) mice were used as bone marrow donors and recipients. β-catenin$^{floxed/floxed}$ and Hoxa9 null mice have been used in our group published data (Yeung et al. 2010) (Smith et al. 2011).

3.2 β-catenin fl/fl, compound Hoxa9 knockout and β-catenin fl/fl mice and Cre mediated deletion of β-catenin

Conditionally-inducible mouse systems were used to assess particular functions of β-catenin in different LSCs after transformation by some of the most frequent LATFs. Cre induced deletion is a powerful tool both *in vitro* and *in vivo* experiments and allows researchers to induce somatic mutations in a tissue-specific and temporary-specific manner (Cheon and Orsulic 2011).

The Cre/loxP inducible system is comprised of two main components: a modified floxed allele ('floxed allele' standing for a gene of interest flanked by two loxP sites) and a conditionally-inducible Cre expression. Cre is 38 KDa integrase that recognizes a 34 base pair sequence called loxP site. These are required for the integration and duplication of bacteriophage genome into and out of the host genome (Sternberg and Hamilton 1981). When Cre is expressed, the recognition between Cre recombinase and loxP sites results in site specific recombination between two loxP sites and mediates a deletion of the floxed portion of the allele. In the ER-Cre system, Cre is induced via tamoxifen to mediate the conditional deletion of a target gene. The way this system works is via the interaction between a nuclear hormone receptor (NHR) such as the estrogen receptor and its antagonist, tamoxifen. In the absence of estrogen or tamoxifen (4OHT), NHR is localized in cytoplasm and suppressed by the binding of heat shock protein 90 (hsp90). When estrogen or tamoxifen are added, NHR is freed from hsp90 segregate, which results in the dimerization and translocation of NHR into nucleus to mediate the expression of genes containing nuclear hormone-responsive promoter. A variation of the system is seen when Cre is fused to the estrogen receptor (ER-Cre). In such a case, after administration of tamoxifen, Cre becomes activated and translocated to the nuclear to mediate gene recombination/deletion by recognizing specific loxP sequence (Garcia and Mills 2002).

Another available tools is self-inactivating hit-and-run Cre, which aims at minimizing long-term toxicity caused by the persistence of Cre expression (Silver and Livingston 2001). The general principle of hit-and-run Cre is as follows: hit-and-run Cre vector contains Cre encoding gene flanked by loxP sites, and this hit-and-run Cre vector gets transduced (*e.g.* retroviral vector) into the host genome that contains the floxed target sequence. Cre gets expressed and mediates the deletion of target genes as well as excising itself (Figure 3.1). Although this system is therefore able to avoid long-term toxicity of Cre expression, it can be technically challenging since only an optimal expression level of Cre can mediate both a complete deletion of target genes and the mediated excision of itself.

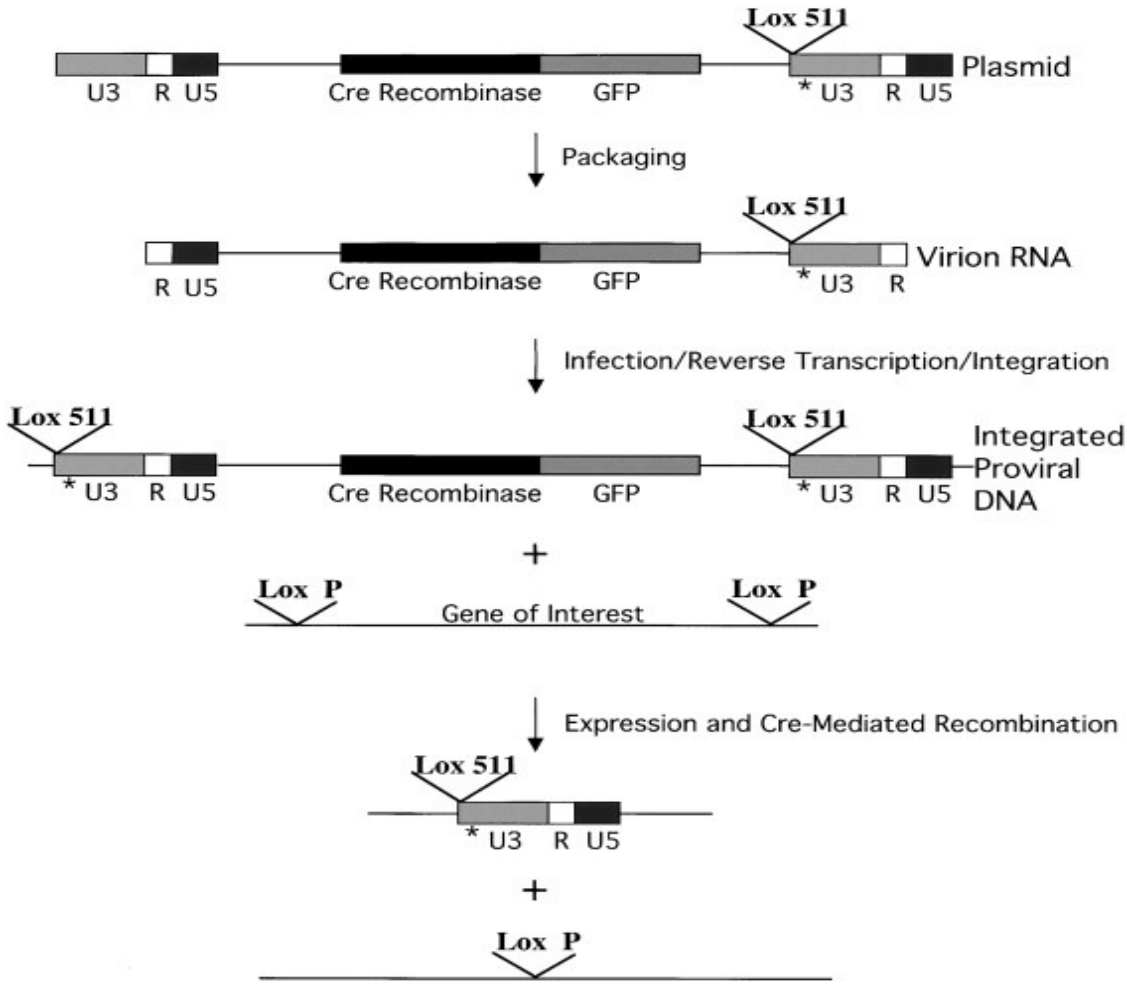


Figure 3.1: Generation of hit-and-run Cre retroviral vector and mechanism of action to mediate the deletion of target sequence (Silver and Livingston 2001).

Initially, in my first experiments, I used the Rosa26-LacZ/Ctnnb1$^{fl/fl}$ (R/C) mouse line that have been used in our group recent published data (Yeung et al. 2010), which has the region between exon 2 and 6 of β-catenin being flanked by loxP sites (Ctnnb1 floxed allele). In addition, a reporter Lac Z sequence has been placed under the control of ubiquitous Rosa26 promoter, with a transcriptional stop flanked by Lox P sites. In the presence of Cre, LoxP sites of both Ctnnb1$^{fl/fl}$ and Rosa26 promoter are cleaved, which allowed Cre to recombine Ctnnb1 (*i.e.* beta catenin) sequence rendering beta-catenin inactive as well as activation of LacZ transcription of β-galactosidase that can be used for monitoring/tracing purposes using a specific substrate (FDG) (Figure 3.2). Assuming that the ability and activity of Cre to mediate the deletion of both target sequences are equal, then, FDG responsive cells will have the completed excision of Ctnnb1 exons 2 to 6 and referred subsequently to as 'β-catenin deficient cells'.

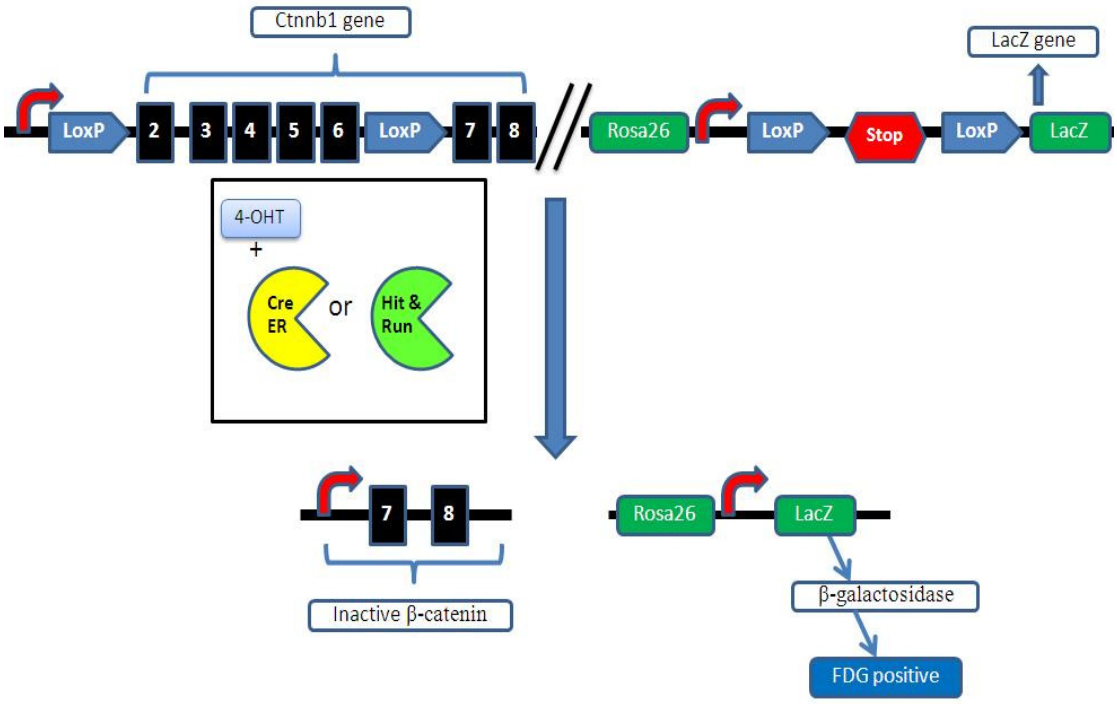


Figure 3.2: Cre mediated deletion of Ctnnb1 floxed allele and of Lac Z promoter region flanked by loxP sites.

In addition to this mouse strain, Rosa26Cre-/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mice have also been generated to overcome a few anticipated potential shortcomings of the R/C mice (Cre leakiness, cell death during FDG staining process). The Cre ER fusion and yellow fluorescent protein YFP (the stop codon being flanked by loxP sites) encoding sequences are placed under the control of two distinct Rosa26 promotors (*i.e.* a single Rosa26 doesn't drive the expression

of both Cre and YFP in this system) (Figure 3.3). The mice were generated after breeding with homozygous Ctnnb1 floxed mice. Administration of tamoxifen results in the Cre mediated deletion of both Ctnnb1 floxed alleles and the stop codon in front of the YFP sequences. Consequently, β-catenin deleted cells can be detected and purified based on the expression of YFP signal. Additionally, this mouse line would allow the assessment of β-catenin function *in vivo* by treating the mice with 4OHT. Areas to be investigated include the requirement of β-catenin for the maintenance of LSC at the onset of the disease. This can be done by directly injecting tamoxifen into recipient mice that were transplanted with transformed Ctnnb1 floxed cells.

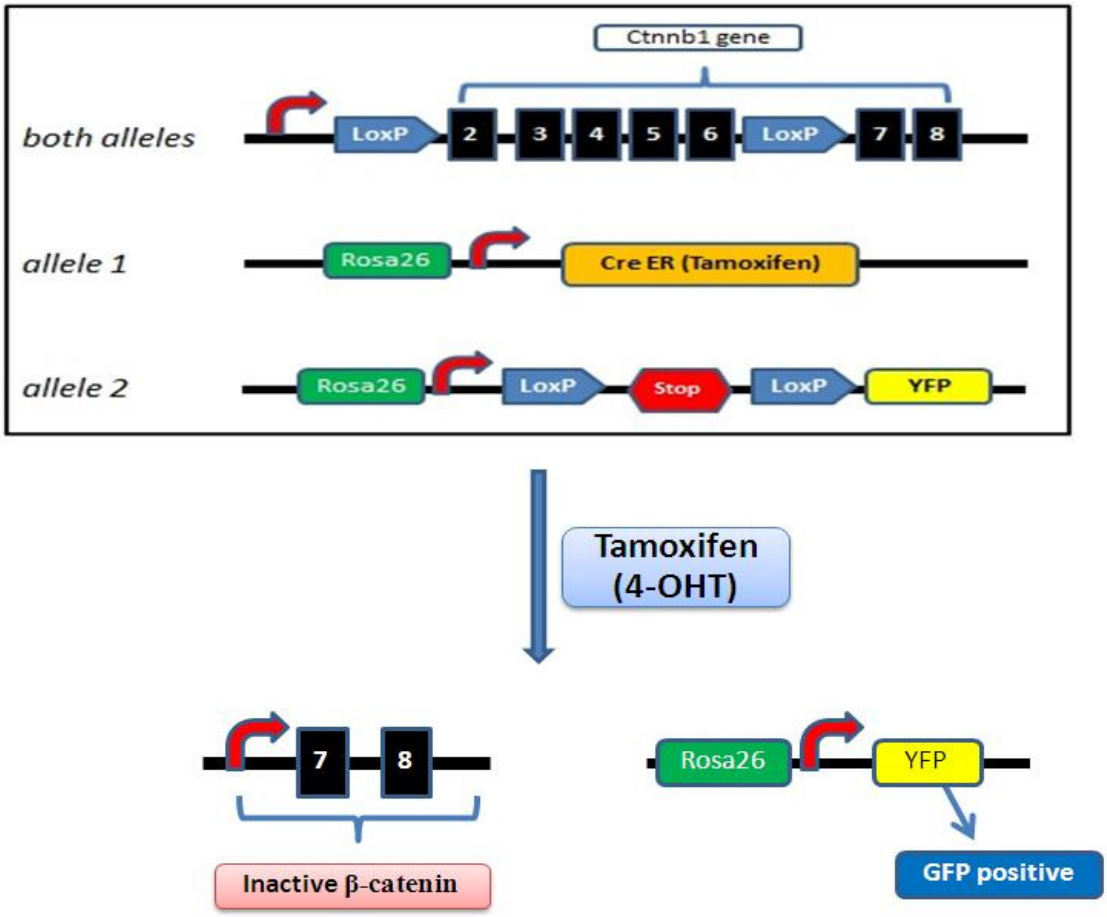


Figure 3.3: Generation of Rosa26Cre/Rosa26YFP/Ctnnb1$^{fl/fl}$ mouse line and tamoxifen mediates deletion of Ctnnb1$^{fl/fl}$ alleles and stop codon of YFP promoter.

Finally, I also generated a compound conditional knockout line with an inducible β-catenin allele (Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$) together with Hoxa9 null alleles which have been used in our group published data (Smith et al. 2011). This mouse line was fertile and showed phenotypically normal when compared to wild type mouse. In the end of this report, I

used this mouse line to further investigate the role of β-catenin and Hoxa9 in the development of leukaemia initiating cells transformed by different LATFs.

3.3 Retroviral transduction/transformation assays (RTTAs)

RTTAs were previously described in detail by our lab (Zeisig and So 2009), here I would briefly describe each step in the following section of the material and methods part of my thesis.

3.3.1 Plasmid and Virus preparation

The murine stem cell virus plasmids (pMSCV) carrying different leukaemia association transcription factors including MLL-AF9, MLL-ENL, AM1L-ETO, E2A-PBX1, E2A-HLF, PML-RARα, Meis1-Hoxa9, MN1, Nup98-Meis1, hit-and-run cre, and pMSCV-IRES-Cre^{TAM}-puromycin plasmids were obtained from the clone database of the Leukemia and Stem Cell Biology group, Haemato-Oncology, King's College London (Kwok et al. 2009, Yeung et al. 2010, Kwok et al. 2010, Smith et al. 2011). All Plasmid DNAs were propagated and prepared using standard plasmid DNA kits from Qiagen/Fermantas according to the manufacturer's instructions. The day before transfection, three million of GP2-293 cells (Clonetech) were seeded in 8 ml of D10 medium (Dulbecco's Modified Eagle Medium (DMEM), with 10 %fetal bovine serum (FBS), 100 IU/ml of penicillin and streptomycin). Transfection were performed using Calcium precipitation with the mixture of 414 µl of H_2O, 15 µg of 1 µg/µl of each DNA construct, 8 µg of 1.0 µg/µl pVSV-G (Vesicular Stomatitis Virus Glycoprotein Epitope), 2 µg of 0.5 µg/µl GFP (Green Fluorescent Protein, Clontech) plasmid, 61 µl of 2M $CaCl_2$, and 500 µl of 2 times HBS. Transfected cells were incubated overnight at 37° C, 5 % CO_2. The transfection efficacy was determined 16 to 18 hours later by monitoring the GFP signal of transfected cells under the fluorescence microscope. Two days after transfection, virus supernatants were collected by aspirating the culture medium with a 10 ml syringe and filtered through a 0.45 µm filter into an ultra-centrifuge tube. Virus was subsequently concentrated using the Optima™ L-100 XP Ultracentrifuge, Beckman Coulter at 25,000 rpm, 4° C for 3 hours. The supernatant was then carefully removed leaving about 3 ml of culture medium at the bottom of centrifuge tube without any disturbance. The concentrated virus supernatant was resuspended by pipetting several times up and down, and stored in 200µl aliquots in 1.5 ml microcentrifuge tubes at -80℃.

3.3.2 Isolation of haematopoietic stem/progenitor cells (c-kit+) from mouse bone marrow

The femurs and tibias bone from R/C and wild type mice were prepared and c-Kit + (CD117) cells were isolated using the MACS, Miltenyi Biotech technology according to the manufacturer's instruction. Briefly, bone marrow from femur and tibias were flushed into 15 ml conical-base tube with 10 ml SM using 10 ml syringe (Terumo®, Belgium) attached with 25G needle (Terumo®, Belgium). Bone marrow cells were centrifuged (Allegra® X-15R centrifuge, Beckman Coulter) at 1300 g, 22 ℃ for 5 minutes. Then the supernatant was carefully removed and the cell pellet was incubated in 1 ml red cell lysis buffer (10 mM $KHCO_3$, 150 mM NH_4Cl, and 0.1 mM EDTA) for 10 minutes at room temperature. After 10 minutes, the red blood cells lysis reaction was stopped by adding 10 ml of SM (phosphate buffer saline (PBS) with 0.2 % FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin) and cells were then filtrated into the 50 ml conical-base tube using 40 µM nylon cell strainer (BD Falcon™) to obtain single cell mononuclear cells. Cells were then centrifuged at 1300 g, 22 ℃ for 5 minutes, washed once with 10 ml SM, reuspended in 500 ul SM and were incubated with 15 µl of c-kit beads for 30 minutes at 4° C. After incubation, cells were washed with 30 ml of SM, while the MS column was pre-equilibrated with 500 µl of SM. After centrifugation, supernatant was carefully removed and cells were resuspended with 500 µl of SM. The cell suspension was applied to the equilibrated MS column, where c-Kit negative cells passed through the column by gravity while c-kit positive were retained. Cells were washed twice with 500 µl of SM in the MS column still attached onto the magnetic stand. The MS column was then removed from magnetic stand and purified c-Kit positive cells were collected onto 1.5 ml sterile microcentrifuge tube by adding 1 ml of SM onto the column and then flushing the column with the plunger into the labelled tube. Cells (20k for single spinoculation or 40k for double spinoculation) were plated in a 96-well plate in 200 ul R10 supplemented with 20 ng/ml SCF and 10 ng/ml of each IL3 and IL6 and incubated overnight at 37℃ with 5% CO2.

3.3.3 Haematopoietic stem cells /progenitors sorting

To sort functionally defined haematopoietic populations I followed and adapted the protocol from Yeung and So, 2009 (Yeung and So 2009). Briefly, bone marrow cells from femurs and tibias of 5-10 mice were collected into 50 ml tube. They were resuspended and filtered through a 40 µm nylon cell strainer. Bone marrow cells were then mixed gently and pelleted by centrifugation at 1300 g, 22 ℃ for 5 minutes. Cell pellet was then resuspended with 1 ml of red cell lysis buffer and incubated at room temperature for 10 minutes. After 10 minutes, 10 ml of SM was added to the cells and the cells were pelleted by centrifugation at 1300 g, 22 ℃ for 5 minutes. Cells were then resuspended in 2.5 ml of SM and incubated with 1 in 40 dilution of unconjugated primary antibody (rat IgG) including Ter119 (TER-119 clone), Mac1 (M1/70 clone), Gr1 (RB6-8C5 clone), CD3e (145-2C11 clone), CD4 (RM4-5 clone), CD8α (53-6.7 clone), and B220 (RA3-6B2 clone) (75 µl of each antibody) at 4℃ for 30 minutes. A 10ul aliquot of unstained cells were also mixed with 400 µl of SM and stored at 4° C for compensation use. Stained cells were then washed once with 10 ml of SM, pelleted at 1300 g, 22 ℃ for 5 minutes and resuspended in 3 ml of SM. A 10 ul aliquot of unsorted cells were also kept at 4° C until use. Dynabead sorting (sheep anti-rat, IgG, Dynal, Invitrogen) was performed according to the manufacturer's instructions. 3 ml of stained cells were incubated with prepared Dynabeads at 4° for 30 minutes (using rotator to allow cells bind to beads efficiently during incubation). Unbound-lineage depleted cells were then purified according to Dynabeads protocol. Lineage-depleted cells were then counted and pelleted by centrifugation as described above. Both Lineage-depleted and Lineage-positive control cells were then resuspended with 1:50 dilution of goat anti-rat-PECy5 in SM at 4℃ for 30 minutes (total volume was 500 µl for lineage-depleted and 200 µl for control/compensation cells). After incubation, stained cells were washed once with 10 ml of SM, pelleted by centrifugation, and supernatant was removed. Then cells were incubated with blocking rat IgG antibody (Sigma) using 250 µl for lineage-depleted and 100 µl for lineage-positive control cells at 4℃ for 30 minutes. Cells were then washed once with 10 ml of SM, pelleted by centrifugation, and supernatant was removed. Cells were then resuspended in master mix of SM and conjugated antibodies including c-Kit-PE (1:100), Sca1-PECy-7 (1:50), CD34-FITC (1:25), and CD16/32-APC (1:75) to make a final volume of 500 µl for lineage-depleted and incubated at 4° C for 30 minutes in dark. Cells were then washed once and pellet as described above. Finally, cells were stained with 1:1000 PI (1mg/ml) in SM/PBS. Stained cells were then subjected to sort HSC, CMP, GMP, and MEP using BD ARIA cell sorter

(BD Bioscience). Sorted cells (0.5 to 2 × 10^4) were seeded in sterile 96-well plate containing with R20/20 supplemented with 20 ng/ml SCF and 10 ng/ml of each IL3 and IL6 and incubated overnight at 37C with 5% CO2.

3.3.4 Colony-forming cell assay in methylcellulose medium

To confirm the purity of HSC/progenitors sorting, I performed the colony-forming cell assay (CFC) to access the proliferation and differentiate patterns of each sorted populations (HSC, CMP, GMP, and MEP) by their ability to form different colonies in semisolid medium. Briefly, complete medium for myeloid lineage cells was prepared by adding 20 ng/ml SCF, 10 ng/ml IL3, 10 ng/ml IL6, 10 ng/ml GM-CSF, 5 unit Erythropoietin, 100 IU/ml penicillin, and 100 µg/ml streptomycin into methylcellulose medium (M03234, StemCell Technology). 1 ml of complete medium was aliquot into 1 ml Eppendorf tubes with appropriate labelled (LSK, CMP, GMP, and MEP) using 18G blunt-end needle attached to a 1 ml syringe. Viable purified LSK, CMP, GMP, and MEP cells were counted using a haematocytometer and 1,000 cells were plated into prepared medium and mixed by vortexing and briefly spun down. A 1 ml syringe attached with 18G blunt-end needle was used to aspirate and to dispense the methylcellulose/cell mix into one well of a 12-well culture plate. Remaining methylcellulose/cell mix was again briefly spun down and transferred into the same well of the 12-well plate. To prevent the methylcellulose medium from drying out during incubation (approximately 5 to 7 days), sterile water or PBS was added into the free first and last row of 12-well plate before putting into the 37° C, 5% CO_2 incubator for 7 to 10 days. After 7 days, colony-forming unit of each sorted HSC progenitors were characterised and numbered based on their discrete in colony morphologies according to Mouse Colony-Forming Cell (CFC) Assays Using Methocult® Technical Manual (version 3.1.1), StemCell Technologies.

3.3.5 Serial replating in methylcellulose medium

To investigate the role of β-catenin in development of LSC induced by different LATF, several transduction strategies using purified c-Kit positive cells and different sorted HSC progenitors (LSK, CMP, GMP, and MEP) were performed. These included; 1) single transduction with only specific LATF; and 2) co-transduction with both specific LATF and Cre virus (hit-and-run/Cre ER) prior the 1st plating. Briefly, frozen concentrated virus was thawed in water bath at 37 ℃ and the centrifuge was pre-warmed to 32℃ prior to spinoculatoin. Most of R10 medium (about 150 µl) was removed from cells in a 96 wells-plate without disturbing the cell pellet on the bottom of the well. Next, 100 µl of thawed and pre-warmed LATF virus for

single spinoculation (or 100 µl of both LATF and Cre viruses for double spinoculation) supplemented with 5 µl of master mix of 4.6 µl R10, 0.1 µl of each 20 ng/ml SCF, 10 ng/ml of IL3, 10 ng/ml of IL6, and polybrene (10mg/ml) was added to the cells and mixed well. Spinoculation was carried out by centrifugation at 800g, 32° C for 2 hours. After spinoculation, cells were placed back into the incubator. The next day methylcellulose medium supplemented with 20 ng/ml SCF, 10 ng/ml IL3, 10 ng/ml IL6, 10 ng/ml GM-CSF, 100 IU/ml penicillin, and 100 µg/ml streptomycin was pre-warmed in waterbath at 37℃. 0.9ml aliquots were dispensed into 1.5ml Eppendorf tubes using 18G blunt-end needle attached to a 1 ml syringe. Antibiotics were added and mixed into medium according to each LATF or Cre-ER construct in an optimal concentration (1mg/ml geneticin, 1 mg/ml hygromycin, and 1.3 µg/ml puromycin for neomycin, hygromycin and puromycin resistant viruses, respectively). For the single spinoculations, transduced cells were transferred into 0.9 ml methylcellulose medium, mixed well by vortexing and briefly spun down. A 1 ml syringe attached with 18G blunt-end needle was used to aspirate and to dispense the methylcellulose/cell mix into one well of a 24-well culture plate (only the 2nd and 3rd row of the 24 well plate were used, see below). Remaining methylcellulose/cell mix was again briefly spun down and transferred into the same well of the 24-well plate. To prevent the methylcellulose medium from drying out during incubation (approximately 5 to 7 days), sterile water or PBS was added into the free first and last row of 24-well plate before putting it back into the 37° C, 5% CO_2 incubator for 5 to 7 days. For co-transduction experiments, spinoculation was repeated once with identical conditions. After a total of two overnight incubations, transduced cells were plated into methylcellulose medium as described above. 5 to 7 days after plating, colony formation of transduced cells was counted under a light microscope. Non-transduced cells were not able to survive and form colonies due to the antibiotic selection. The number and type of colonies were scored and recorded. Colonies were harvested in 8 ml SM and transferred into a 15 ml conical base test tube using a 10 ml pipette. Cells were incubated in 37℃ water bath for 1-2 hours to dissolve methylcellulose before they were centrifuged at 1300 g, 22 ℃ for 5 minutes. Cells were reuspended in 500 to 1,000 µl of SM depending on initial numbers of colonies (e.g., 1 ml for more than 100 colonies, 500 µl for less than 100 colonies). Viable cells were counted using a haematocytometer, and 10000 cells were re-plated into 0.9 ml methylcellulose supplemented with cytokines as described above for the first round of plating. Five to seven days after the plating, the numbers as well as colony morphologies were scored and recorded again. Cells were then subjected to a third round of plating using the

same method as described above. This re-plating was carried out up to 8 times. It is important to point out that only transformed cells with enhanced the self-renewal property form colonies in the third round of plating and beyond, while non-transformed cells do form colonies in the initial round of plating but fail to form colonies in the third round of plating as they differentiate and stop proliferating. Transformed cells were then immunophenotypically analyzed using FACS (described on immunophenotyping section) and cell lines of each transformed cell was established by growing transformed cells in R20/20 supplemented with the same cytokine mix as in methylcellulose re-plating assay.

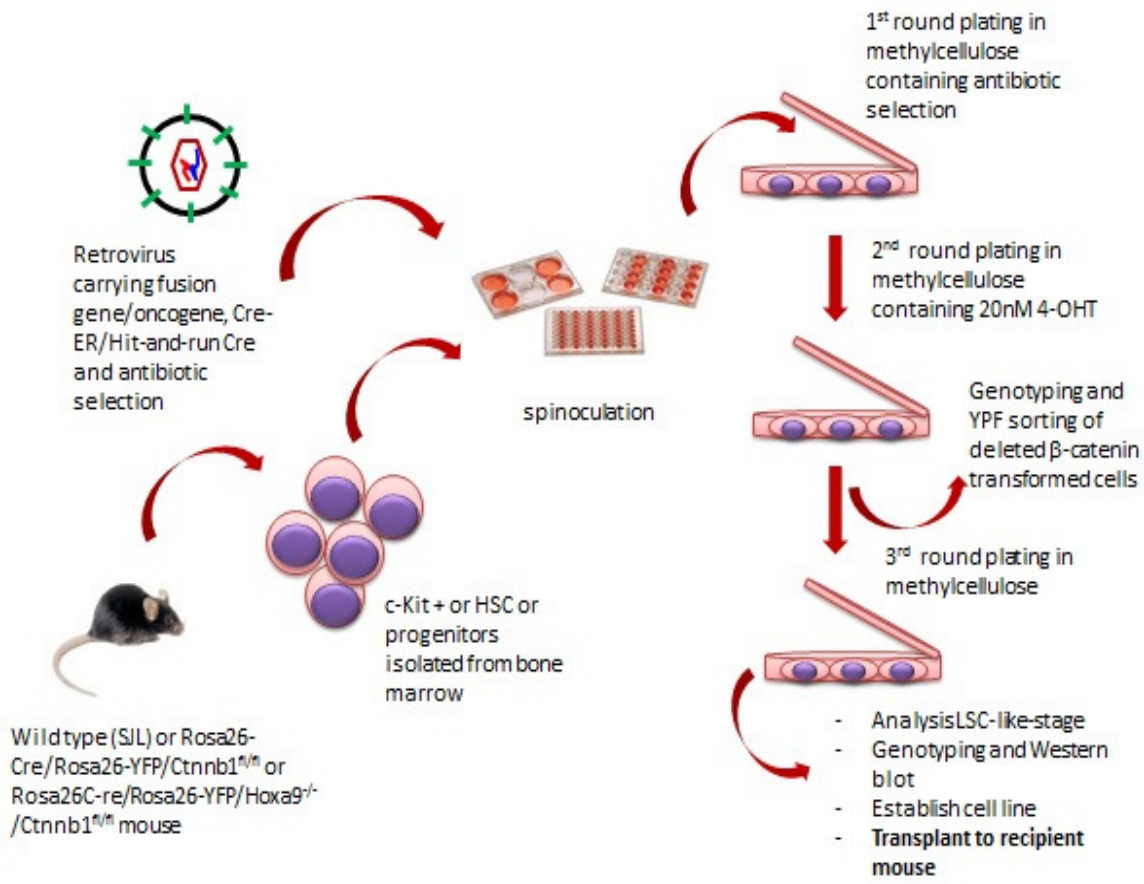


Figure 3.4: Schematic demonstrates a modified retroviral transduction and transformation assays (RTTA) which is central to my studies.

3.4 Mouse β-catenin and Hoxa9 genotyping

To determine and confirm Ctnnb1 and Hoxa9 genotypes of haematopoietic progenitor cells were isolated from bone marrow of Rosa26-LacZ/Ctnnb1$^{fl/fl}$, Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$, Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$/Hoxa9$^{-/-}$, and wild type mice before and after transduced with each LATF and Cre systems. For Ctnnb1 genotyping, allele specific PCR according to Brault et al (2001) (Brault et al. 2001) was performed using 0.5 µM of each primers (Table 3.1 and figure 3.5), 1X Dream Green buffer (Fermentas), 0.2 mM dNTP mix (Roche), 0.5 unit of DreamTag™ Green DNA Polymerase (Fermentas) using the following PCR condition: denaturing at 94 ℃ for 4 minutes, 30 cycles of 94 ℃ for 30 seconds, 56 ℃ for 30 seconds, 72 ℃ for 30 seconds, final extension at 72 ℃ for 30 seconds. The specific amplified Ctnnb1 alleles (wild type; 221 bp, floxed; 324 bp, and deleted; 500 bp) were separated on 1.5% agarose gel. Similar PCR cocktail was used for both Hoxa9 wild type and knockout genotyping. Primers specific amplified to Hoxa9 wild type and knockout alleles were listed in table 3. PCR conditions of both Hoxa9 wild type and knockout were following denaturing at 94 ℃ for 4 minutes, 30 cycles of 94 ℃ for 30 seconds, 62 ℃ for 30 seconds, 72 ℃ for 30 seconds, final extension at 72 ℃ for 30 seconds. Specific PCR products (wild type approximately to 140 bp and knockout approximately to 100 bp) were separated on 1.5% agarose gel.

Table 3.1: Table shows Ctnnb1 and Hoxa9 specific primers.

Primer	Sequence (5' to 3')
RM41	AAG GTA GAG TGA TGA AAG TTG TT
RM42	CAC CAT GTC CTC TGT CTA TTC
RM43	TAC ACT ATT GAA TCA CAG GGA CTT
Hoxa9 Wild type Forward	CAC AAA GGG GCT CTA AAT CC
Hoxa9 Wild type Reverse	AGC ACA TAC AGC CAA TAG CG
Hoxa9 K/O Forward	AAG GCA GGT CAA GAT CTC CGA
Hoxa9 K/O Reverse	TCG CCT TCT TGA CGA GTT CTT

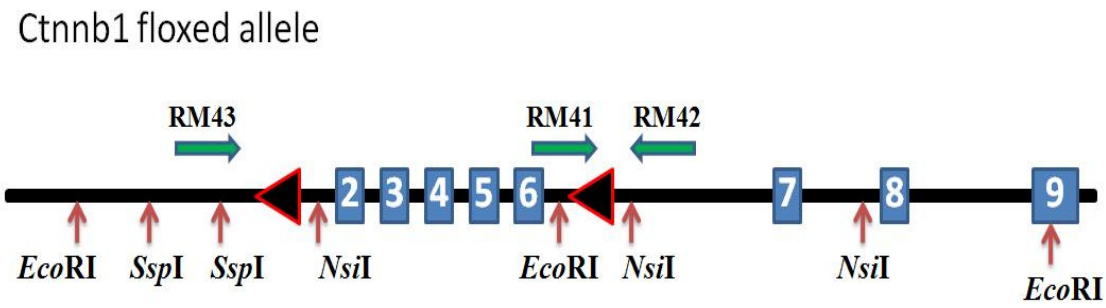


Figure 3.5: Schematic illustrates Ctnnb1 floxed allele and the primers binding site (applied from Brault et al. (Brault et al. 2001).

3.5 Isolation of LacZ positive cell using Fluorescence-activated cell sorting (FACS)

The Rosa26-LacZ/Ctnnb1$^{fl/fl}$ (R/C) mice are homozygous for the Ctnnb1 allele flanked by loxP sites, and possess the Rosa26 LacZ transgene regulated by a transcriptional stop signal flanked by loxP sites (figure 10). As described in the RTTA assay, the haematopoietic progenitors isolated from bone marrow of both R/C and wild type mice were co-transduced with viruses carrying LATFs and Hit and Run Cre or the inducible Cre ER viruses. When the Cre was expressed from either the transient Hit and Run Cre or the inducible Cre-ER (mediated by Tamoxifen (4-OHT) incubation), it would delete the Ctnnb1 gene and remove the transcriptional stop in the Rosa26 LacZ reporter. To isolate cells that transiently expressed Cre, LacZ expressing cells were sorted using the FDG (Fluorescein Digalactoside) as a fluorogenic substrate for β-galactosidase which is encoded by LacZ gene.

Briefly, to prepare 20 mM stock FDG solution, a 1:1 mixture of 38 µl DMSO and 38 µl ethanol was prepared and added to dissolve 5 mg FDG powder (Molecular Probes, Invitrogen). Next, 304.4 µl of ice cold H_2O was added to the solution to make 20 mM of 8:1:1 for H2O:DMSO:ethanol FDG stock solution. They were stored as 20ul aliquots at -20 ℃ in dark. For cell staining, a 20 µl aliquot of 20 mM FDG was diluted with 180ul sterile 37° C H_2O to make a 10x working FDG solution. Cells were counted and up to 1 X 10^6 cells and resuspended in 50 µl of culture medium or SM. 50 µl of 10x working FDG solution was added into the cells suspension and immediately incubated for exactly 1 minute in 37° C wate rbath. Then 1 ml of

ice cold medium or SM was quickly added, and incubated on ice for 30-60 minutes. Cells were then centrifuged at 1300 g, 22℃ for 5 minutes, supernatant was removed, and washed once again with ice cold SM. Cells were resuspended in 500 µl of SM medium containing a 1:1000 dilution of 1 mg/ml propidium iodide and transferred into sterile FACS tube with lids and placed on ice until sorting. Fluorescein positive and negative cells were sorted using the BD FACSAria Special Order System, BD Biosciences (figure 3.6). Finally, sorted cells were counted and plated out in methylcellulose medium to continue the serial replating assay.

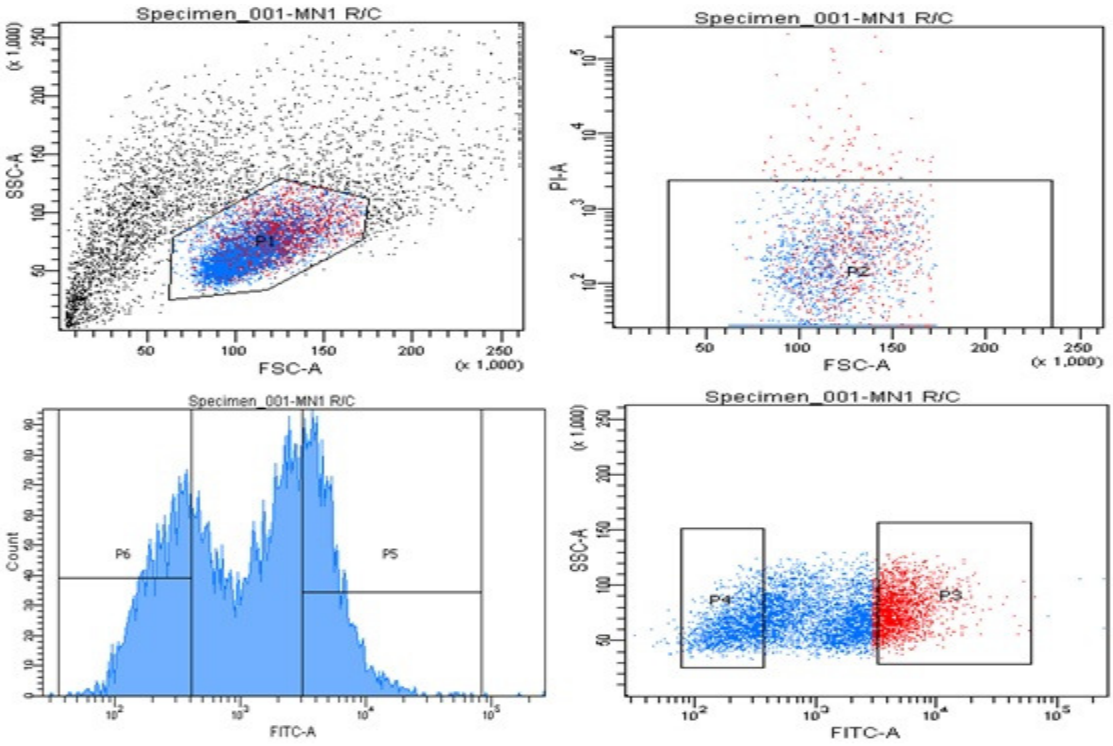


Figure 3.6: FDG positive cells sorting after Cre mediated deletion of β-catenin. FACS shows GFP positive (P3 and P5 gates) and GFP negative cells (P4 and P6 gates) after FDG staining and those cells were subjected to sorting, assuming that GFP positive cells were β-catenin deleted cells.

3.6 Cre-ER mediated deletion of β-catenin in Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ and Rosa26-Cre/Rosa26-YFP/Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ and the Isolation of YFP positive cell using Fluorescence-activated cell sorting (FACS)

Taking the advantage of the two new mouse lines, Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ and Rosa26-Cre/Rosa26-YFP/Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ with stably integrated ER-Cre and floxed YFP alleles in Rosa26 loci, I aimed to overcome the shortcomings associated with the R/C line and viral expression of Cre such as (1) Cre leakiness, (2) induction issue, and (3) cell death during FDG staining. Furthermore, another advantage of these new mouse line is the ability to reliably mediate β-catenin deletion after disease onset by tamoxifen administration in vivo. Different haematopoietic cell populations isolated from Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ and Rosa26-Cre/Rosa26-YFP/Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ mice were transduced with different LATFs such as MLL-AF9 and MLL-ENL and subjected for RTTA (as described in chapter 3.2.5). After the first round, transformed cells were serially replated into two different replating conditions; 1) second round without tamoxifen and 2) second round with 20nM tamoxifen. At this round, Cre-ER mediated deletion of β-catenin was occurred in transformed cells with tamoxifen treatment as well as subsequently activated the expression of YFP signal. Thus, the number of YFP positive cells was represented to the amount of transformed cells with β-catenin deletion or vice versa. To purify the YFP positive/β-catenin deleted cell, I used the fluorescent cell activated sorting (FACS) technology. In brief, second round number of colony and colony morphologies were scored and recorded in both control and tamoxifen treatment cells. Colonies from both control and tamoxifen treated cells were harvested in 8 ml SM and transferred into a 15 ml conical base test tube using a 10 ml pipette. Cells were incubated in 37℃ water bath for 1-2 hours to dissolve methylcellulose before they were centrifuged at 1300 g, 22 ℃ for 5 minutes. Cells were washed once with 10 ml of SM by pipetting up and down several times and centrifuged at 1300 g, 22 ℃ for 5 minutes. Cells were resuspended in 300 to 500 µl of 1:1000 PI (1mg/ml) in SM/PBS depending on initial numbers of colonies (e.g., 300 ml for less than 100 colonies, 500 µl for more than 100 colonies). YFP positive cells were sorted using the BD FACSAria Special Order System, BD Biosciences (figure 3.7). Finally, sorted cells were counted and plated out in methylcellulose medium to continue the serial replating assay.

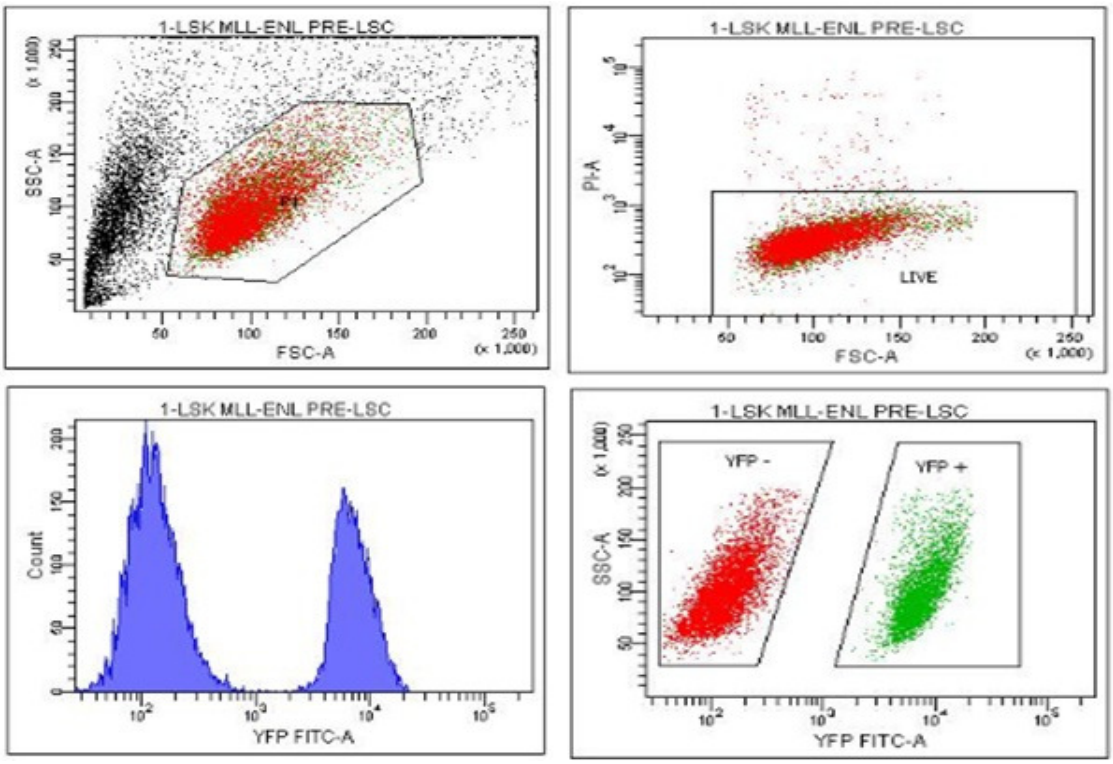


Figure 3.7: YFP positive cells sorting after Cre mediated a deletion of β-catenin. FACS shows YFP positive (YFP+) and YFP negative cells (YFP-) after 2nd round of replating with 20 nM tamoxifen and those cells were subjected to sorting, assuming that YFP positive cells were β-catenin deleted cells.

3.7 Cell culture

Primary transformed cells were cultured in R20/20 (RPMI1640, 20% FCS, 20% WEHI, 100 U/ml penicillin, and 100 µg/ml streptomycin). WEHI, a conditioned medium (source of IL-3) was prepared by growing WEHI cells in R10 for three days. WEHI media was then harvested using 0.22 µm filter and frozen at -20° C until use. Medium was replaced or replenished when the colour changed to yellow before cultured cells grew too dense. Generated cell lines were then frozen in cryotubes by resuspending them in freezing medium (90% FCS and 10% DMSO) and stored in -80℃ or in liquid nitrogen.

3.8 Western blotting

To confirm the β-catenin knockout, western blotting was performed according to Yeung et al. 2010 (Yeung et al. 2010). 1-2 × 10^6 cells/ml were washed with phosphate buffer saline (PBS) for three times. Then cells were lysed by adding 2X sample buffer at 1 × 10^6 cells/100 µl.

Lysate samples were sonicated to shear DNA and to reduce sample viscosity by the Diagenode, Bioruptor® at high pulse for 5 minutes. After sonication, samples were placed at 95 to 100 ℃ for 5 minutes. 20 µl of each protein sample and 10 µl of protein ladder (PageRuler™ Plus Prestained Protein Ladder, Fermentas) were separated on 10% gradient SDS polyacrylamide gels (NEXT GEL™, Amresco) at 150 V for 80 minutes, then blotted onto a nitrocellulose membrane (Amersham™ Hybond™ ECL) at 100 V for 1 hour. The transferred membrane was blocked with 2.5% milk dissolved in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) for 30 to 60 minutes at room temperature (RT). Then membrane was probed with a 1 in 2,000 dilution of mouse anti-β-catenin antibody (clone 14/Beta-Catenin, BD) for 1 hour at RT or overnight at 4℃. After probing the membrane with the primary antibody, it was washed with PBS-T (5 minutes, 4 times) and incubated with secondary antibody, peroxidise-conjugated goat anti-mouse IgG (heavy and light chains (Jackson ImmunoResearch Laboratory, INC.) or the goat polyclonal IgG β-actin antibody conjugated with HRP (Actin (I-19; SC-1616) HRP, Santa Cruz Biotechnology) for 1 hour at room temperature. Then the membrane was washed with PBS-T (5 minutes, 4 times). Chemiluminescent signal of conjugated antibodies were detected by using ECl Western Blotting Detection System, Amersham™, GE Healthcare, UK. according to a manufacturer's instructions. Then signals were developed on X-Ray film (Amersham Hyperfilm™ ECL) using the Photon imaging system. The expected size of β-catenin and β-actin (control protein) proteins are 92 kDa and 43 kDa, repectively.

3.9 Immunophenotyping using FACS

Cells were harvested after the third round of plating, 10^4-10^5 cells in a volume of 200ul were stained in FACS tubes with master mix of FACS antibodies including c-Kit (D7 clone), Mac-1 (M1/70 clone), Gr-1 (RB6-8C5 clone), B220 (RA3-6B2 clone), CD4 (RM4-5 clone), and CD8a (53-6.7 clone) in FACS tubes (1ul of each antibody per sample, mixed well keep in dark before use). One unstained control tube contained only 200 µl of 10^4-10^5 cells in PBS/medium without any antibody. Sample tubes were incubated at 4℃ in the dark for 10-30 minutes. After incubation, samples were then washed twice with 4 ml PBS/SM by centrifugation at 1,300 g, 22° C for 5 minutes. They were then resuspended in 400 µl of SM with 1 µg/ml propidium iodine. Finally, the expression of cell surface markers was analyzed using a LSRII flow cytometer (BD Bioscience).

3.10 Cell cycle analysis using propidium iodide

Cells were harvested after the third round of plating, 10^6 were washed twice with cold PBS and resuspened in 300 µl of cold PBS in FACS tubes. Cells were fixed by adding 900 µl of cold absolute ethanol by dropwise while vortexing. Fixed cells were incubated in fridge for at least 1 hour. Cells were washed twice with cold PBS and resuspended in 300 µl of propidium iodide staining solution (3.8 mM sodium citrate, 40 ug/ml PI (Sigma, P 4170) in PBS) and subsequently added 15 µl of RNase A stock solution (10µg/ml) and incubated in fridge for 3 hours. Finally, the cell cycle profile was analyzed using a LSRII flow cytometer (BD Bioscience).

3.11 Transplantation (in vivo leukaemogenic assay)

Transplantation experiment was performed according to our previous reports (Yeung et al. 2010, Smith et al. 2011). Briefly, five to ten mice per condition were sub-lethally irradiated with 1,100 rad in split doses. I transplanted a mixture of 10^5-10^6 of pre-LSCs for primary and 10^2-10^5 LSCs for secondary transplantation together with 2 x 10^5 rescue total bone marrow cells isolated from bone marrow of a mouse with carrying the same CD45 (CD45.1 or CD45.2) marker was prepared in 150ul PBS for transplantation. At least 1 hour after the 2nd irradiation, recipient mice were placed one by one into a cylindrical mouse restrainer for tail vein injection. Finally, transplanted mice were returned into their cage.

When the transplanted mouse showed signs of sickness (e.g. loss of condition and/or paralyze legs), mouse was then sacrificed and organs including spleen, liver and bone marrows were taken for analysis. FACS analysis for CD45 markers could be used to distinguish donor from recipient cells. Moreover, using a panel of antibodies as described in 3.8 allows determining the immunophenotype of leukemic cells and comparisons with the immunophenotype of the original transplanted cells. Note that leukemic mice usually display enlarged spleen and pale/white bone marrow, and leukaemic cells commonly infiltrate to other organs such as liver.

3.12 RNA sequencing

To analyse the transcriptional changes and targets critical for β-catenin and/or Hoxa9 functions, high throughput RNA sequencing was performed on different RNA samples extracted from different MLL-ENL transformed cells. Briefly, RNA from 1-5 x 10^6 different pre-LSCs or 2 x 10^4 to 2 x 10^5 normal haematopoietic stem/progenitor cells were extracted using mirVana™ miRNA Isolation Kit, Life Technologies, according to a manufacture's instruction. Quality and amount of extracted RNA was determined using the Agilent 2100 bioanalyzer (Agilent Technologies, Clara, California). 0.1-1 µg of total RNA depleted of rRNA, were fragmented to generate cDNA library with appropriated RNA index (adaptor), and enriched cDNA fragments using TruSeq Stranded Total RNA LT Sample Prep Kit, Illumina® according to according to a manufacture's instruction. cDNA fragments were clustered, sequenced, and analysis using using HiSeq™ 2000 Sequencing System, Illumina® at the Department of Haematological Medicine**,** King's College London.

RNA sequencing data was analysed by Mr. Sam Tung Hoi, PhD student who is specialised in bioinformatics. In brief, Sequenced reads were aligned to mouse genome (mm9) using TopHat version 2.0.9 (Kim D 2013). Differential expression analysis was performed using DESeq2, Bioconductor DESeq2 v1.2.10 software (Love MI 2014) and Limma, Limma v.3.18.13 software (GK 2005). Gene Set Enrichment Analysis (GSEA) was performed using Pre-ranked tool version 2.0.13 with 10,000 permutations. A full list of genes with the logarithmic fold change from DESeq2 or Limma was input into GSEA as gene ranking metric. A normalized enrichment score (NES) for each gene set was calculated by Kolmogorov-Smirnov-like statistic and the significance level of NES was computed to yield an estimated false discovery rate (FDR) (Subramanian A 2005).

3.13 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA from purified HSC, CMP, and GMP and different pre-LSCs were extracted using mirVana™ miRNA Isolation Kit, Life Technologies or NucleoSpin RNA Isolation Kit, Macherey-Nagel (MN) according to a manufacturer's instructions. Total RNA concentration was measured by using NanoDrop 8000 instrument, Thermo Fisher Scientific. cDNA was synthesised by using SuperScript III (Invitrgen) followed by PCR using Veriti® Thermal Cycler, Applied Biosystems®. In brief, 10 µl master mix of 1-5 µg RNA, 1 µl of 50 µM oligo $(dT)_{20}$, and 1 µl of 10 mM dNTP was incubated at 65° C for 5 minutes then place on ice for at least 1 minute. cDNA synthesis master mix was prepared by adding 4 µl of 10X RT buffer, 2 µl of 50 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNaseOUT™ (40 U/µl), and 1 µl of SuperScript® III RT (200 U/µl). 10 µl of cDNA synthesis master mix was added to each RNA/primer mix, gently mix, and briefly spun down. cDNA synthesis condition was following 50 °C for 50 minutes, 85 °C for 5 minutes, and hold at 4 °C. Real time PCR was performed to quantify the expression level of interested genes (table 3.2 and table 3.3) using Fast SYBR® Green Master Mix or TaqMan® Fast Universal Master Mix on the StepOnePlus™ Real-Time PCR System, Applied Biosystems®. Finally, RNA expression data was normalized to GAPDH and represented as a fold change.

Table 3.2: Primers pairs for qRT-PCR using SYBR green detection reagents

Target genes	Sequences
Mouse *Ctnnb1*	5-ATGGAGCCGGACAGAAAAGC-3’
	5’-CTTGCCACTCAGGGAAGGA-3’
Mouse *Mpl*	5’- TCACCTTGGTGACTGCTCTG -3’
	5’- CCACAAAGCATGCCTCAGTC -3’
Mouse *Cebpα*	5’-GCA AAG CCA AGA AGT CGG TG-3’
	5’-CAC CTT CTG TTG CGT CTC CA-3’
Mouse *Gata1*	5’- CTC TCA TCC GGC CCA AGA AG -3’
	5’- AGC TTG AAA TAG AGG CCG CA -3’
Mouse *Mmp8*	5’- CTTTCAACCAGGCCAAGGTA-3’
	5’- GAGCAGCCACGAGAAATAGG-3’
Mouse *Mmp9*	5’-TGTCATCCAGTTTGGTGTCG-3’
	5’-AATGGGCATCTCCCTGAAC-3’
Mouse *Ruvbl2*	5’-TCCGAGAAGGGAAGATTGC-3’
	5’-TGGCTGTGAATGGTGTGTC-3’
Mouse *Prmt1*	5’-ACCCTCACATACCGCAACTC-3’
	5’-CAGCAAACATGCAGAGGATG-3’
Mouse *Ruvbl1*	5’-AGACAGAGGTGCTGATGGAGA-3’
	5’-GGGGTTCTCTGTCTCACAGG-3’
Mouse *Nolc1*	5’-CAAAGCTTCAGAGAGCAGCA-3’
	5’-GTCTGACTGCCTTGGCTTG-3’
Mouse *C1qbp*	5’-TCCCAAGATGTCTGGAGATTG-3’
	5’- GTTGGAGGGATGCTGTTGTT-3’
Mouse *Tgfbr3*	5’-TCCCAGAAGCTGCCAAAGTGT-3’
	5’-GACTCCTTCATGTTTGGAACA-3’
Mouse *Irf4*	5’-CTACCCCATGACAGCACCTT-3’
	5’-TGACTGGTCAGGGGCATAAT-3’
Mouse *Tgm2*	5’-GATGACCAGGCCAGACCTAC-3’
	5’-CGAAGGGTGCATCATACTTG-3’

Table 3.3: Primers pairs for qRT-PCR using TaqMan® detection reagents (Dickson GJ 2009).

Target genes	Primers and probes sequences
Mouse *Hoxa5*	5'-TCCCATCGCTTCCCTACCT-3' 5'-GCTTTGGAACAGCCTACAGCTT-3' FAM-ACCCATTGCAAAGTTCAGGGCATAAGGT-TAMRA
Mouse *Hoxa7*	5'-AGCTTGGAAATTCTGCTCACTTCT-3' 5'-TCTGATGTCATGGCCAAATTTG-3' FAM-ACCCATTGCAAAGTTCAGGGCAT AAGGT-TAMRA
Mouse *Hoxa9*	5'-GCCGGCCTTATGGCATTAA-3' 5'-CAGGGACAAAGTGTGAGTGTCAA-3' FAM-TGAACCGCTGTCGGCCAGAAGG-TAMRA
Mouse *Hoxa11*	5'-TTGAGCATGCGGGACAGTT-3' 5'-GTACCAGATCCGAGAGCTGGAA-3' FAM-AGGCGCTTCTCTTTGTTAATGTAGACGCTGAA- TAMRA
Mouse *Hoxb4*	5'-CCCTGGATGCGCAAAGTT-3' 5'-AATTCCTTCTCCAGCTCCAAGA-3' FAM-AAGCGCTCTCGGACCGCCTACAC-TAMRA
Mouse *Meis1*	5'-CCTCGGTCAATGACGCTTTAA-3' 5'-TTTGAGAAATGTGAATTAGCTACTTGTACC-3' FAM-ACACCCCCTCTTCCCTCTCTTAG CACTGA-TAMRA

3.14 Prmt1 Knockdown

shRNAs constructs including GFP-tagged PRMT1 shRNA and GFP-tagged vector control were previously published by our group (Cheung et al., 2007) and obtained from the clone database of the Leukemia and Stem Cell Biology group, Haemato-Oncology, King's College London. Plasmids and viruses were prepared as described in section 3.2.1. 1-2 x 10^6 LSC cells were resuspended in 3 ml of fresh R20/20 and transferred into each individual well of 6 wells culture plate (control and test wells). 1 ml of concentrated viruses including GFP-tagged PRMT1 shRNA and GFP-tagged vector control and 3 µl polybrene (10mg/ml) were added into each well with appropriate labelled, mixed by pipette up and down several time. Spinoculation was carried out by centrifugation at 800g, 32° C for 2 hours. After spinoculation, cells were top up with 4 ml of R20/20 and placed back into the incubator. The day after, transduced cells were, determined the number of GFP positive cells, and sorted GFP positive cells by FACS. Transduced cells were expanded in R20/20, extracted RNA to measure the expression of Prmt1 by qRT-PCR, and studied the impact of Prmt1 knockdown in different LSCs in both in vitro colony forming assay and in recipient mouse.

3.15 Statistical analysis

In this study, number of colonies during each transformation assays were manually counted under microscope following with the calculation of mean and standard deviation using Microsoft excel (2007). In real time quantitative RT-PCR (RQ-PCR) assays, Ct (cycle threshold) values of each sample was normalised with GAPDH control gene and calculated to relative gene expression using Microsoft excel (2007). The log-rank test and Gehan-Breslow-Wilcoxon test were used to compare survival curves. LDA plots were performed by using the limdil function of package statmod (Smyth G 2010). The p value less than 0.05 were considered as statistically significant.

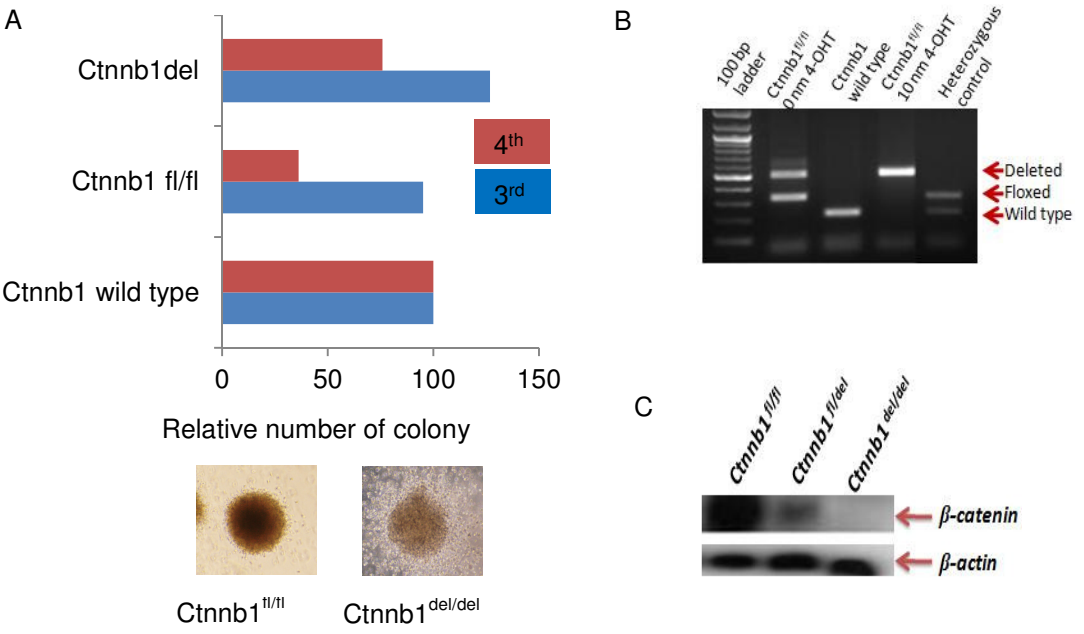
Chapter 4 Results

4.1 The role of β-catenin in transformation of haematopoietic stem/progenitor (c-kit+) cells by various LATFs

During the generation of a conditionally inducible Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mouse, I used the Rosa26-LacZ/Ctnnb1$^{fl/fl}$ (R/C) mouse to investigate the role of β-catenin in the development of AML LSCs transformed by different LATFs such as MLL-ENL, MLL-AF9, E2A-PBX1, and MN1. The preliminary data of my initial experiments using c-Kit+ cells isolated from bone marrow of R/C mouse are described below.

4.1.1 Establishment of c-Kit+ E2A-PBX1 LSCs is independent of β-catenin

After optimising the experimental conditions such as the amount of CreER virus and the concentration of tamoxifen, I was able to achieve a complete deletion of Ctnnb1 using 10nM tamoxifen in the second round of replating of c-kit+ cells from R/C mice co-transduced with Cre-ER and E2A-PBX1 (Figure 4.1) as confirmed by PCR and Western blot (Figure 4.1B and 4.1C). Interestingly, E2A-PBX1 expressing cells with a complete β-catenin deletion showed similar number of colonies (figure 4.1A), colony morphology (figure 4.1A), and immunophenotype (figure 4.1D) when compared with E2A-PBX1 transformed wild type cells, suggesting that β-catenin is dispensable for the establishment of E2A-PBX1 pre-LSCs.



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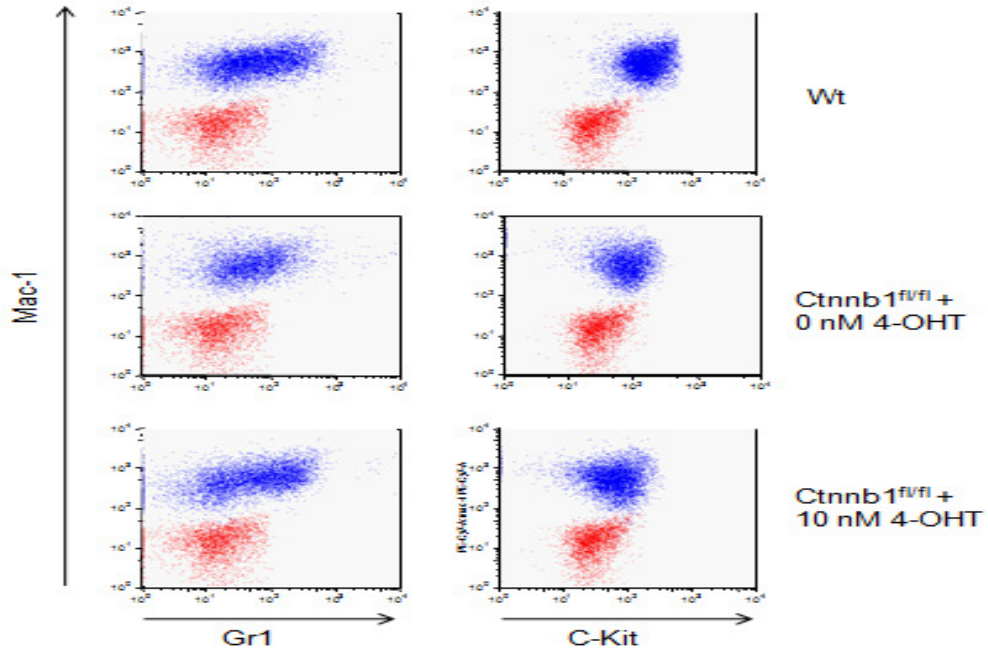


Figure 4.1 Establishment of pre-LSCs by E2A-PBX1 is independent of β-catenin. A) Relative colony numbers (top) and colony morphologies (bottom) of c-Kit+ cells transformed by E2A-PBX1 during replating assay. B) and (C) PCR genotyping of Ctnnb1 status and Western blot using antibodies specific for β-catenin and β-actin on E2A-PBX1 pre-LSCs from the indicated genotypes. D) Immunophenotypic analysis using flow cytometry of wild type (top), Ctnnb1$^{fl/fl}$ (middle) and Ctnnb1$^{del/del}$ (bottom) E2A-PBX1 pre-LSCs.

To further investigate whether β-catenin is required for E2A-PBX1 LSC development in vivo, E2A-PBX1 pre-LSCs derived from Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ genetic backgrounds were transplanted into syngeneic C57BL6 mice for disease development. 2 of 5 (40%) mice that received either Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ E2A-PBX1 pre-LSCs developed leukaemia with very similar latencies (p = 0.8985) (Figure 4.2). PCR genotyping of Ctnnb1 status of leukemic cells confirmed the presence of the floxed or the deleted Ctnnb1 alleles accordingly (Figure 4.3A and 4.3B). The results strongly suggest that β-catenin is not required for the development of E2A-PBX1 LSC derived from c-Kit+ cells.

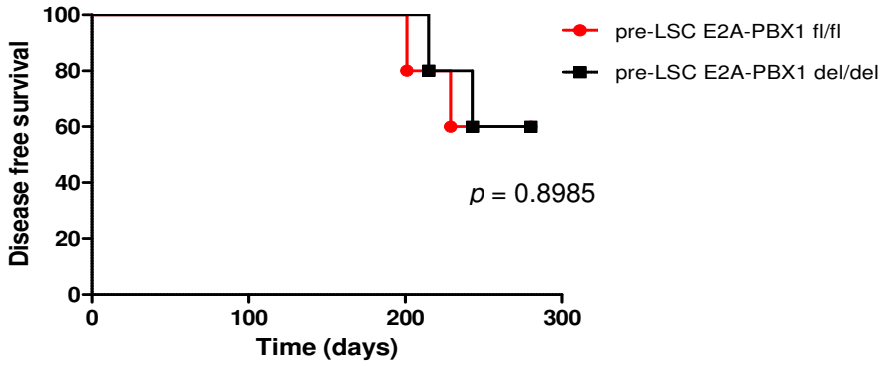


Figure 4.2: Establishment of E2A-PBX1 is β-catenin independent. Survival curves of mice transplanted with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ E2A-PBX1 pre-LSC (n=5 for each group).

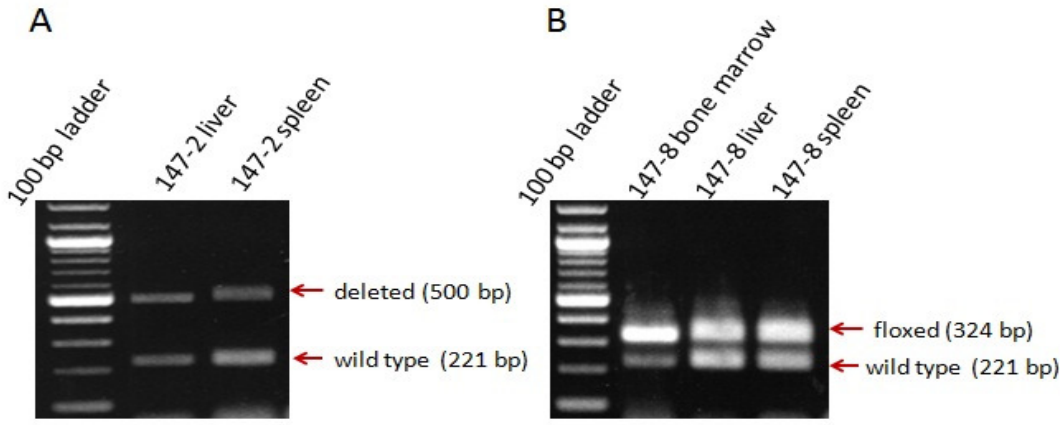


Figure 4.3: PCR genotyping of Ctnnb1 status on genomic DNA isolated from mouse transplanted with E2A-PBX1 pre-LSC. (A) 147-2 and (B) 147-8 which were transplanted with Ctnnb1$^{del/del}$ and Ctnnb1$^{fl/fl}$, E2A-PBX1 pre-LSCs, respectively.

After I initiated my studies using R/C line, this model suffered from a number of shortcomings associated with viral expression of Cre such as (1) Cre leakiness/toxicity due to the level/copy of Cre expression, (2) induction issue, and (3) cell death during FDG staining. Thus after my successful generation of the new mouse line Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$, which can be reliably mediate β-catenin deletion even after disease onset by tamoxifen administration in vivo, I switched all my experiments using this much improved model. The obtained results using c-Kit+ cells isolated from bone marrow of this new mouse line to investigate the role of β-catenin in the development of AML LSCs transformed by MN1 and MLL-ENL are described below.

4.1.2 c-Kit+ MN1 LSCs is β-catenin independent

To test the feasibility of using Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mouse, I investigated the functional requirement of β-catenin in mouse AML initiated by MN1 oncogene which is commonly overexpressed in AML. In in vitro transformation assay, knockout of β-catenin in c-Kit+ MN1 pre-LSCs did not affect the cloning efficiency and colony morphology when compared to the c-Kit+ MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ (figure 4.4).

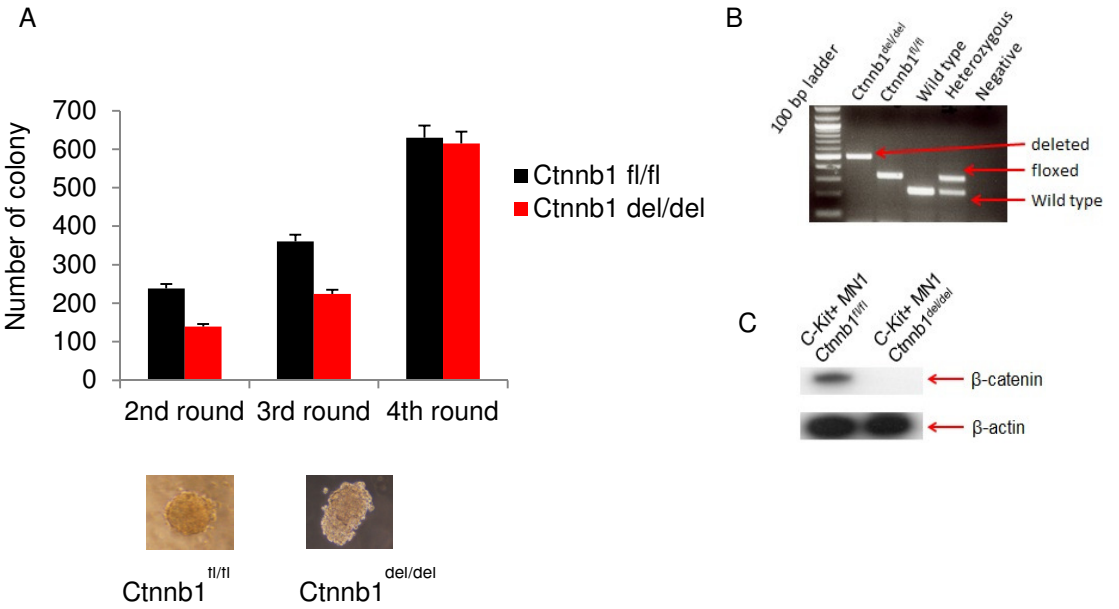


Figure 4.4: Establishment of c-Kit+ MN1 pre-LSC is independent of β-catenin. A) Absolute colonies number (top) and colony morphologies (bottom) of c-Kit+ cells transformed by MN1 during replating assay. B) Genotyping of Ctnnb1 on gDNA extracted from c-Kit+ MN1 transformed cells after CRE-ER mediated deletion of Ctnnb1 gene with 20nM tamoxifen treatment and YFP sorting. C) Western blot shows a complete deletion of β-catenin in c-Kit+ MN1 transformed cells.

To investigate the role of β-catenin in the development of MN1 LSCs in vivo, these pre-LSCs were transplanted into syngeneic recipient mice (n=5 for each group). All mice (100%) that received c-Kit+ MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ (n=7) or Ctnnb1$^{del/del}$ (n=8) developed leukaemia with very short latency (p = 0.0401) (median survival = 36.7 and 48.2 days, respectively). Similar to primary transplantation, secondary recipient mice that were transplanted with either MN1 with Ctnnb1$^{fl/fl}$ or MN1 with Ctnnb1$^{del/del}$ LSCs rapidly showed signs of leukaemia with median survival of 13 and 19.4 days, respectively (figure 4.5). Together the findings revealed that transformation of c-Kit+ MN1 LSC is β-catenin independent.

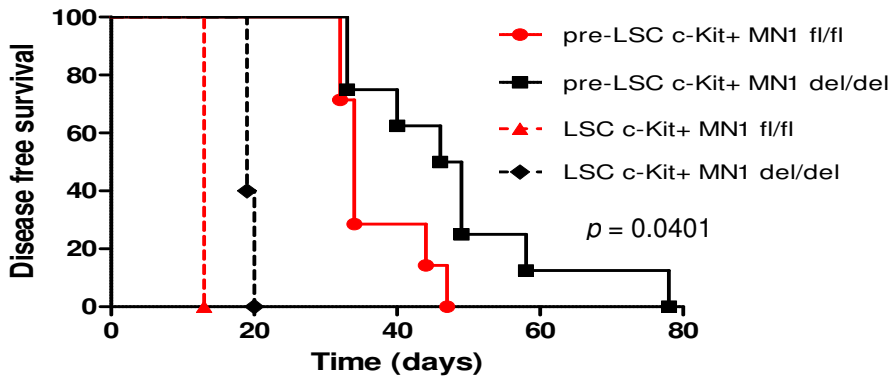


Figure 4.5: β-catenin is not required for the initiation of c-Kit+ MN1 LSC. A) Survival curve of mice transplanted with c-Kit+ MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ (n = 7), c-Kit+ MN1 pre-LSCs with Ctnnb1$^{del/del}$ (n = 8), LSCs c-Kit+ MN1 with Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ (n=5 for each group).

4.1.3 β-catenin is critical for the development of c-Kit+ MLL-ENL LSCs

To functionally validate the new mouse line, I sought to confirm published data from our group using R/C line with MLL-ENL (Yeung et al. 2010). Purified c-Kit+ cells from bone marrow of Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mouse were transduced with pMSCV-MLL-ENL-puromycin virus and subjected to RTTA. Although β-catenin was conditionally deleted at the early phase of the initiation of MLL-ENL pre-LSC by adding 20 nM tamoxifen during the second round of plating, MLL-ENL transformed c-kit+ cells in the absence of β-catenin exhibited similar cloning efficacies and colony morphologies as compared with pre-LSC MLL-ENL with Ctnnb1$^{fl/fl}$ cells (figure 4.6).

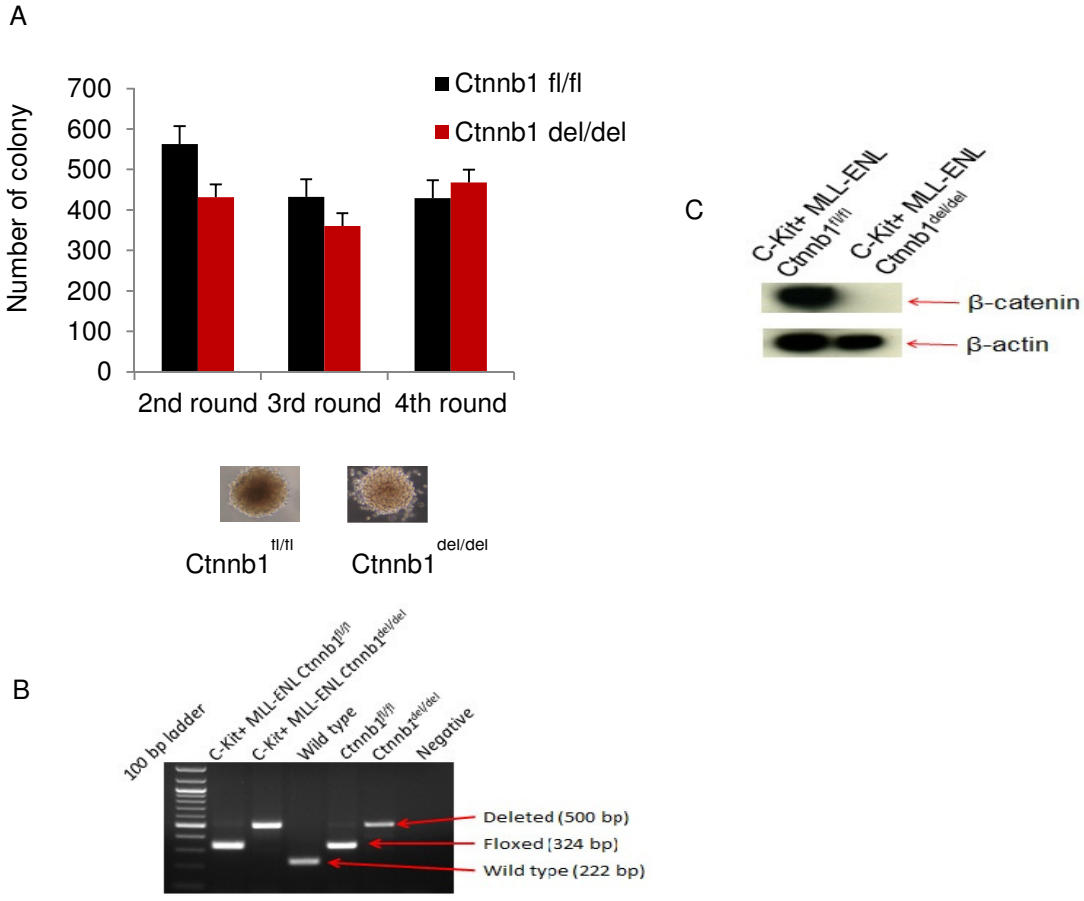


Figure 4.6: In vitro transformation of c-Kit+ MLL-ENL pre-LSC is independent of β-catenin. A) Absolute colony numbers (top) and colony morphologies (bottom) of c-Kit+ cells transformed by MLL-ENL during replating assay. B) Genotyping of Ctnnb1 on gDNA extracted from c-Kit+ MLL-ENL transformed cells after deletion of the Ctnnb1 gene with 20 nM tamoxifen and YFP sorting. C) Western blot shows a complete deletion of β-catenin in c-Kit+ MLL-ENL transformed cells.

For in vivo studies, I serially transplanted these pre-LSC cells into sub-lethally irradiated syngeneic mice. Mice that received c-Kit+ MLL-ENL pre-LSCs with Ctnnb1$^{fl/fl}$ cells developed leukaemia in primary and secondary transplanted mice (median survival = 85.5 and 21.6 days, respectively) (figure 4.7). In contrast, c-kit+ MLL-ENL pre-LSC with the complete deletion of β-catenin could not induce leukaemia ($p < 0.001$) and could not be detected in recipient mice when mice were sacrificed 150 days after transplantation.

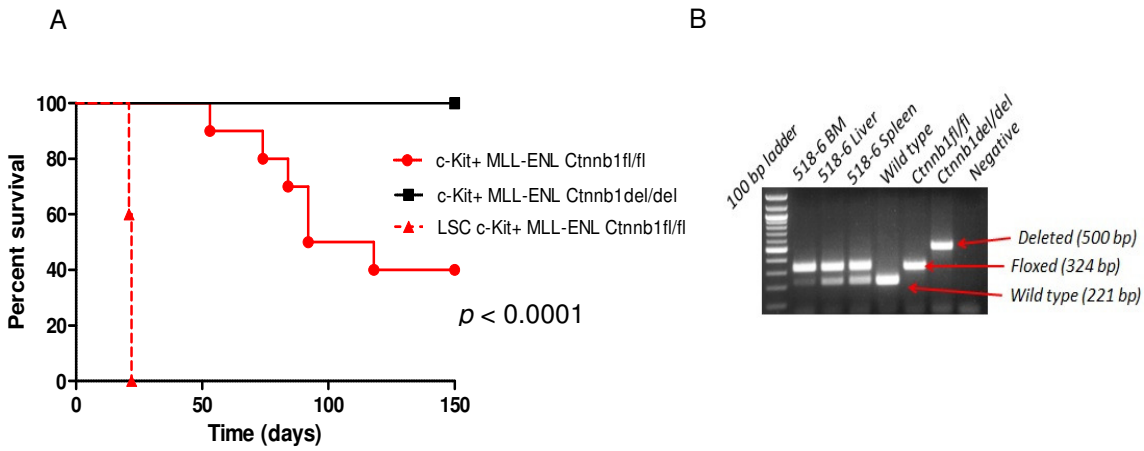


Figure 4.7: Knockout of β-catenin abolishes oncogenic property of c-Kit+ MLL-ENL pre-LSC. A) Survival curve of mice transplanted with c-Kit+ MLL-ENL pre-LSCs with Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ (n=10 for each group) and LSC c-Kit+ MLL-ENL with Ctnnb1$^{fl/fl}$ (n=5). B) Genotyping of Ctnnb1 on gDNA extracted from bone marrow, spleen, and liver of leukaemic mouse that received pre-LSC c-Kit+ MLL-ENL.

Collectively, these results not only confirmed our previously published data obtained with a different mouse model for an important role of β-catenin in the development of full-blown MLL-ENL LSC derived from haematopoietic stem/progenitor enriched, c-Kit+ cells, but also demonstrate the feasibility and utility of this new Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mouse line.

4.2 HSC/progenitors purification and their gene expression profiles

In contrast to c-kit+ cells, which are a mixed population of stem and progenitor cells, defined haematopoietic populations can be isolated from mouse bone marrow that have been functionally and phenotypically described (Yeung and So 2009). To this end, bone marrow cells from wild type (SJL) and Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mice (n = 5 for each group) were sorted (by fluorescent activated cell sorting (FACS) technology) based on their surface marker (CD protein) expression. . While Lin$^-$ c-Kit$^+$ Sca-1$^+$ CD34$^+$, LSK cells were enriched for HSCs with self-renewal property, non-self-renewing CMP and GMP were Lin$^-$ c-Kit$^+$ Sca-1$^-$ CD34$^+$ CD16/32$^{low/-}$ and Lin$^-$ c-Kit$^+$ Sca-1$^-$ CD34$^+$ CD16/32$^{high/+}$, respectively. From the pooled total bone marrow cell of 5 mice, I could purify approximately 2 to 3 million lineage negative cells (lineage depleted cells; Ter119$^-$, Mac1$^-$, Gr1$^-$, CD3$^-$, CD4$^-$, CD8$^-$, and B220$^-$), which contained an average between 70,000 to 90,000 LSK, 200,000 to 300,000 CMP, 400,000 to 600,000 GMP, and 90,000 to 100,000 MEP cells. In addition to post-sort analysis, which revealed around 90 % purity for the individually sorted populations, I also functionally determined the purity of HSC/progenitors sorting. To this end, I performed colony forming cells assay (CFS) to classify and enumerate the different colony types resulted from LSK, CMP, GMP and MEP to by plating 1,000 cells of each purified population into methylcellulose medium supplemented with mouse cytokines including SCF, IL3, IL6, GM-CSF, and Erythropoietin followed. After incubation at 37° C, 5% CO2 for 5 to 7 days, LSK cells mainly gave rise to Colony-forming unit-granulocyte macrophage (CFU-GM, 95.8%) and also in low frequency to Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM, 1.4%), Colony-forming unit- macrophage (CFU-M, 2.4%) and Colony-forming unit-granulocyte (CFU-G, 2.4%) but not to Burst-forming unit-erythroid (BFU-E). CMPs gave rise to predominantly CFU-GM (74.8%), CFU-GEMM (19.9%), CFU-M (3.9%), CFU-G (1.4%), but not to BFU-E. GMPs, as more mature progenitors gave mainly rise to CFU-GM (37.8%), CFU-G (34.4%) and CFU-M (25.6%) and in much lower frequency to CFU-GEMM (2.2%) but not to BFU-E. In contrast, MEPs predominantly gave rise to BFU-E (75.0%) followed by CFU-G, CFU-M, and CFU-GEMM 8.3% each) but not to CFU-GM (figure 4.8). Although in this work I used different mouse strains and slightly different culture conditions including methylcellulose and supplemented mouse cytokines from previous reports (Akashi 2000, Lieu 2012), similar distributions (in percentage) of classified colony forming unit

arisen from each sorted LSK, CMP, and GMP were observed. Thus, I could confirm the functional properties of HSC/progenitors sorting by colony forming cells assay (CFC).

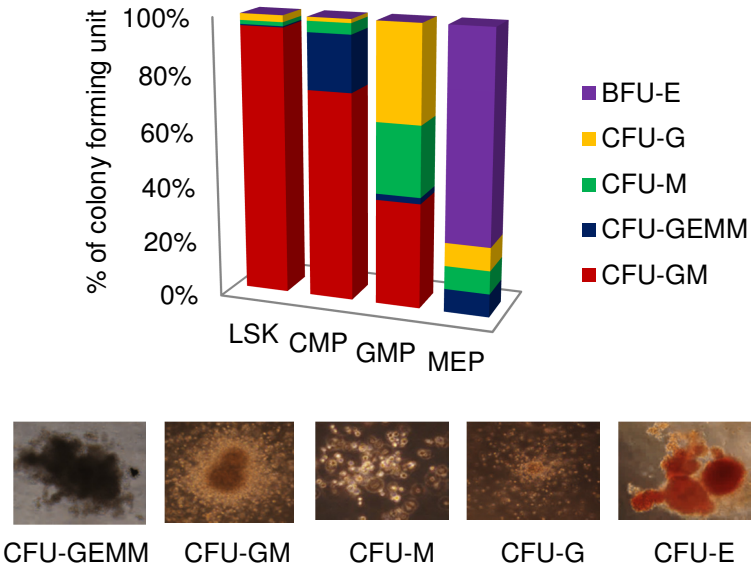


Figure 4.8: Colony forming cells assay (CFC) of purified HSC (Scheller et al.), CMP, GMP, and MEP. Distribution (top) and morphology (bottom) of the different colony forming units (CFUs) (in %) outgrown of sorted HSC (Scheller et al.) and progenitors plated into methylcellulose medium. Data were obtained from two independent experiments.

To molecularly confirm the purity of sorted cells and to study the gene expression signatures of HSC/progenitors, RNA from around 70,000-100,000 cells per purified populations was isolated and subjected to qRT-PCR (quantitative-reverse transcription-polymerase chain reaction). Well defined, population specific markers differently expressed in normal HSC and progenitors were used to assess the purity of isolated cells. I was able to detect appropriate expression of various markers including *Mpl* (highly expressed in HSCs, then decreases through haematopoietic differentiation) (JC 2011), *Cebpα* (Highly expressed in GMP, low levels in HSC) (Zhang P 2004), and *Gata1* (highly expressed in MEP, low levels in CMP) (Gutiérrez L 2008) . As a result, I also molecularly confirmed the purity of sorted cells based on the different expression of lineage specific gene expression of isolated HSC/progenitor cells (figure 4.9).

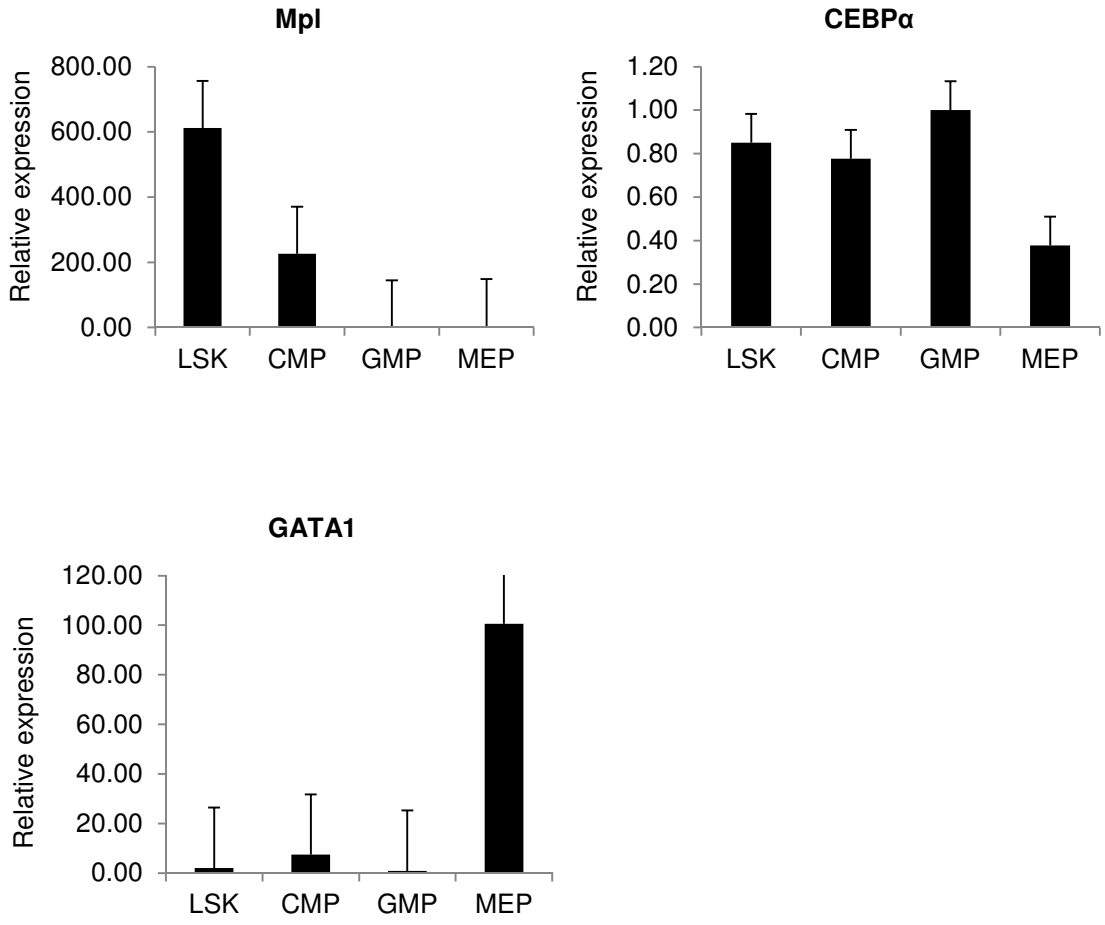
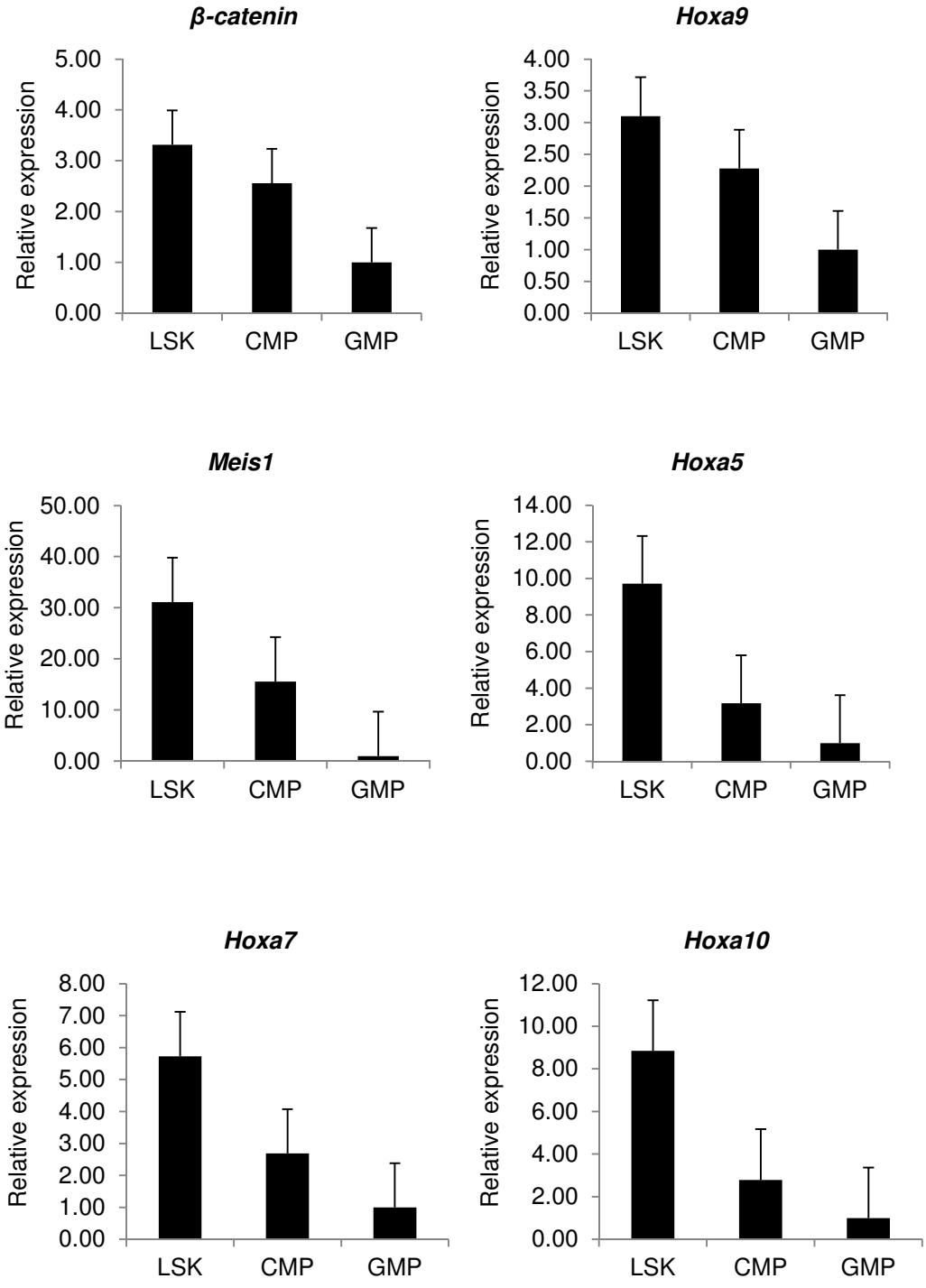


Figure 4.9: Different expression of *Mpl*, *Cebpα*, and *Gata1* in LSK, CMP, GMP, and MEP isolated from wild type mouse.

To further study the gene expression profiling of HSC/progenitor cells, I next focused on expression levels of genes that are critical for self-renewal pathways in both normal haematopoietic stem cells as well as leukaemia stem cells including *Ctnnb1* (β-catenin), *Hoxa9*, *Meis1*, *Hoxa5*, *Hoxa7*, *Hoxa10*, *Hoxa11*, and *Hoxb4*. I could demonstrate that these genes were highly expressed in LSK (HSC-enriched) cells which can self-renew, whereas their expression levels progressively decrease during haematopoietic differentiation (figure 4.10). To get high resolution global gene expression profiles and to establish novel data sets of gene expression signatures in normal mouse HSC/progenitors, RNA from sorted haematopoietic populations have been being subjected to high throughput RNA sequencing although the data will not be included in this thesis. Together I demonstrated high purity of sorted HSC/progenitor cells on several levels including functional (by using colony forming cells assays) and molecular analyses (qRT-PCR on lineage specific gene expression markers). Furthermore, I also

demonstrated high mRNA expression levels of critical self-renewal genes such as β-catenin, Hoxa9, and Meis1 in normal HSCs as compared to their downstream progenitors.



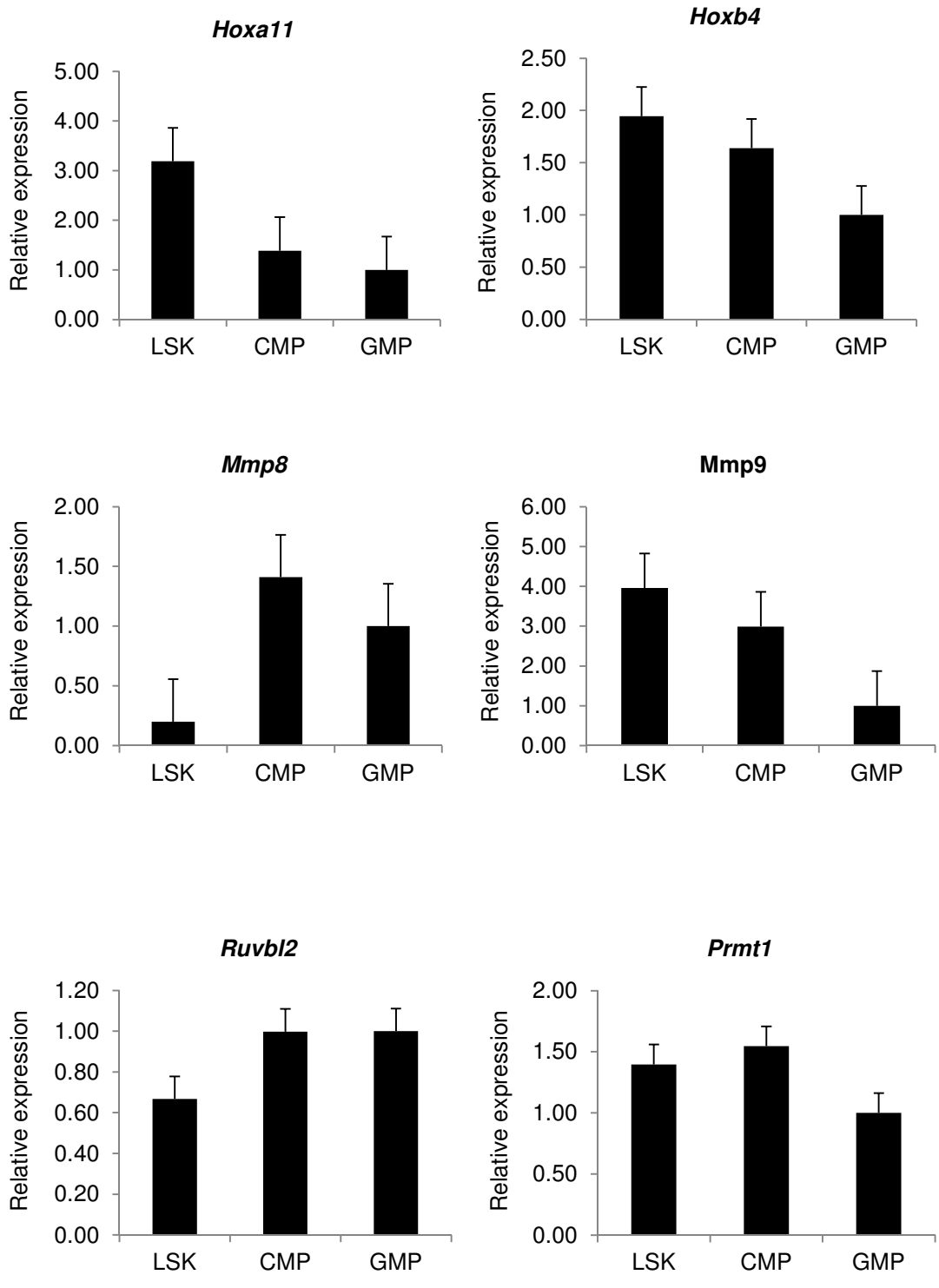


Figure 4.10: Different gene expressions in LSK, CMP, and GMP isolated from bone marrow of wild type mice. Similar genes expression patterns were observed in two independent experiments.

4.3 The impact of β-catenin in generating and maintaining LSCs from different haematopoietic cell populations

To define the impact of cellular origins in utilization of self-renewal pathways in LSCs, I investigated the functional requirement of β-catenin in different cellular origins transformed by various LATFs. To this end, I have purified HSC-enriched $Lin^{-}Sca^{+}Kit^{+}$ (Scheller et al.), common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) for standard RTTA. In my initial experiments using the Rosa26-LacZ/$Ctnnb1^{fl/fl}$ (R/C) mouse, both Hit-and-run Cre and MEIS1-HOXA9 were co-transduced into different sorted populations isolated from wild type and R/C mice for RTTA. After the second round of plating, cells were stained with FDG (based on LacZ activity) to assist the isolation of Ctnnb1 (β-catenin) successfully deleted cells for continuation of RTTA as described in the previous section. In addition, I also used purified HSC, CMP, and GMP cells isolated from the new mouse line, Rosa26-Cre/Rosa26-YFP/$Ctnnb1^{fl/fl}$, to assess the different functional β-catenin requirement of in different cell of origins transformed by MN1 and MLL-ENL. I will describe the results for each individual LATFs tested starting with MEIS1-HOXA9 using the R/C line.

4.3.1 LSK but not CMP required β-catenin for the development of LSC transformed by Meis1-Hoxa9

To assess the ability of Meis1-Hoxa9 to transform LSK, CMP and GMP populations from both wild type and $Ctnnb1^{fl/fl}$, I carried out RTTA experiments in which Meis1-Hoxa9 was transduced into the different sorted cell populations. As shown in Figure 4.11, Meis1-Hoxa9 competently transformed all populations from both genotypes, which resulted in similar number of colonies and colony morphology.

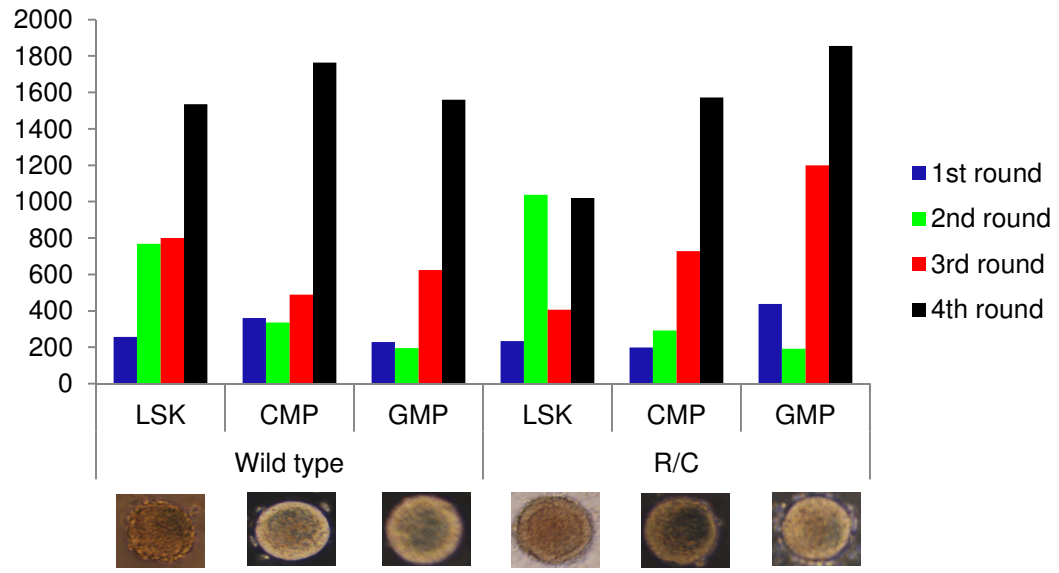


Figure 4.11: Establishment of LSK-, CMP-, and GMP-Meis1-Hoxa9 pre-LSC. Bar chart (top) show the absolute number of colonies in each round of plating in Meis-Hoxa9pre-LSCs originated from the indicated cell populations. Typical 3rd round colony morphology are shown (bottom).

Next I tested the requirement of β-catenin Meis-Hoxa9 mediated transformation by co-transducing hit-and-run Cre and Meis-Hoxa9 into different cell populations. FDG positive cells were isolated by FACS after the second round of plating, and sorted cells were again subjected to RTTA. As a result, Ctnnb1$^{del/del}$ Meis-Hoxa9 expressing LSKs (Figure 4.12C, D) formed smaller, more diffused and fewer numbers of colonies than their Ctnnb1$^{fl/fl}$ counterparts in all rounds of plating (Figure 4.12A, B). In contrast, CMPs transformed by Meis-Hoxa9 did not show any significant difference in terms of colony number and morphology in RTTA (Figure 4.13A, B) regardless of β-catenin status (Figure 4.13C, D), suggesting a distinctive functional requirement for β-catenin in different cell populations. Thus β-catenin may be critical for establishment of LSK pre-LSC, but is dispensable for CMP pre-LSCs induced by Meis-Hoxa9.

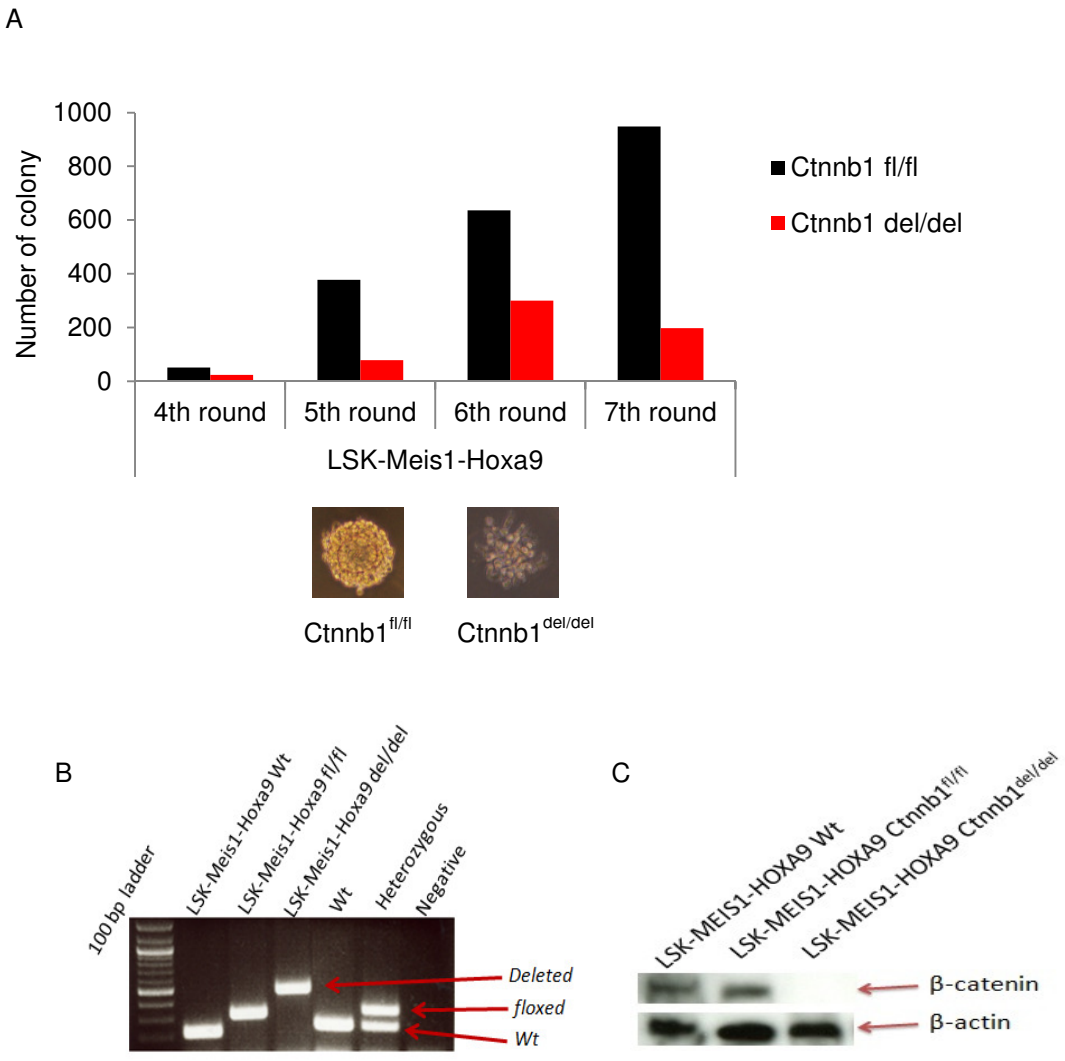


Figure 4.12: Knockout of β-catenin suppresses leukaemogenic potential of LSK-Meis1-Hoxa9 pre-LSC. A) Absolute colonies number and colony morphologies of LSK cells transformed by Meis1-Hoxa9 during replating assay. B) Genotyping of Ctnnb1 on gDNA extracted from LSK-Meis1-Hoxa9 transformed cells after CRE-ER mediated deletion of Ctnnb1 gene by 20 nM tamoxifen treatment and YFP sorting. C) Western blot shows a complete deletion of β-catenin in LSK-Meis1-Hoxa9 transformed cells.

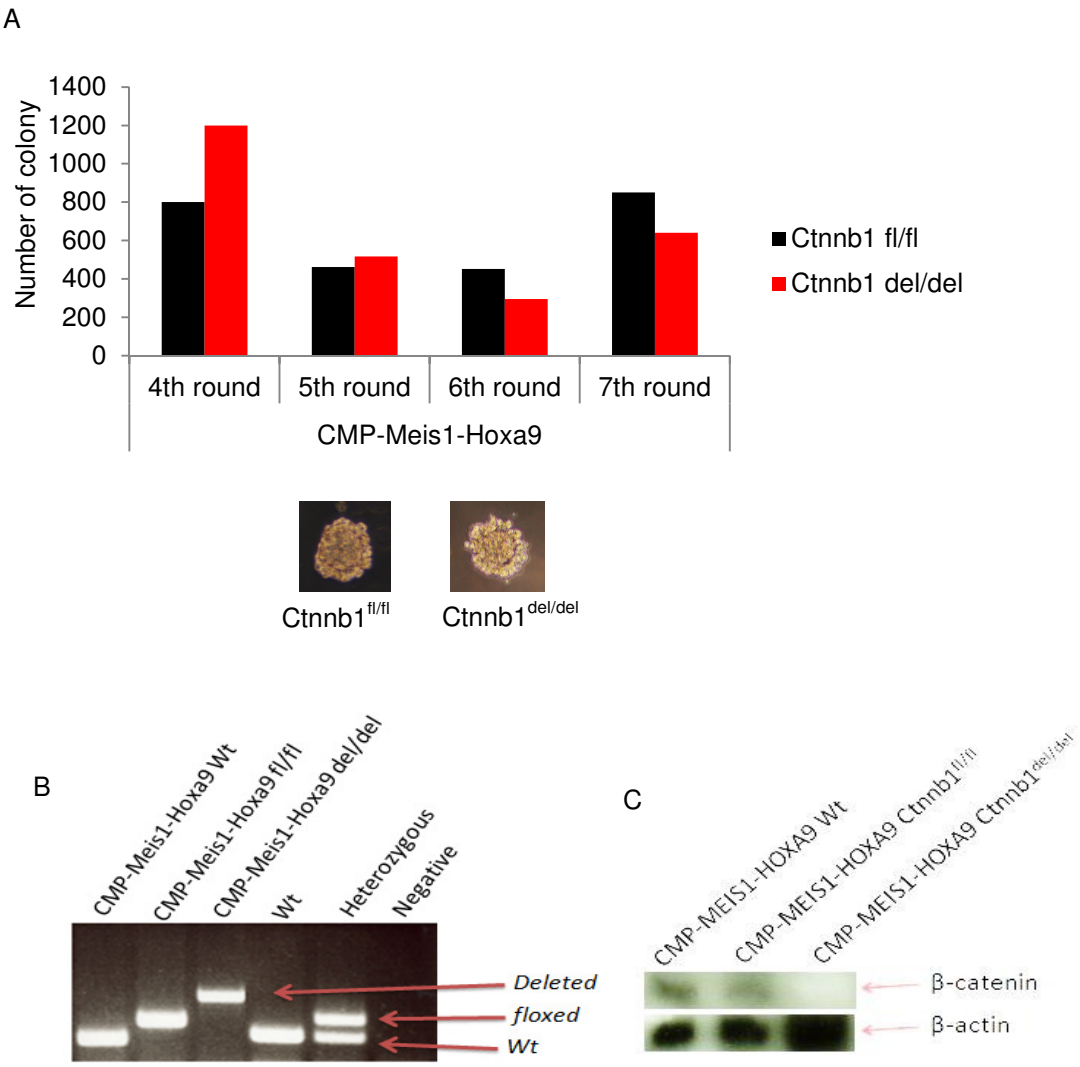


Figure 4.13: Establishment of CMP-Meis1-Hoxa9 pre-LSC is β-catenin independent. A) Absolute colonies number and colony morphologies of CMP cells transformed by Meis1-Hoxa9 during replating assay. B) Genotyping of Ctnnb1 on gDNA extracted from CMP-Meis1-Hoxa9 transformed cells after Cre-ER mediated deletion of Ctnnb1 gene by 20nM tamoxifen treatment and YFP sorting. C) Western blot shows a complete deletion of β-catenin in CMP-Meis1-Hoxa9 transformed cells.

To further investigate whether β-catenin is required for development of Meis1-Hoxa9 LSCs in vivo, LSK and CMP pre-LSCs from wild type, Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ were transplanted to primary recipient mice (n=5 for each group) for disease development. While all of the mice transplanted with LSK-Meis1-Hoxa9 pre-LSCs from wild type background (n=5) and 4 of 5 (80%) mice received Ctnnb1$^{fl/fl}$ LSK-Meis1-Hoxa9 pre-LSCs developed leukaemia, mice transplanted with Ctnnb1$^{del/del}$ LSK-Meis1-Hoxa9 did not show any sign of disease (p = 0.0133)

(Figure 4.14A). Consistently, PCR genotyping of leukaemic cells confirmed their β-catenin status (Figure 4.14B). In contrast, mice transplanted with both Ctnnb1$^{fl/fl}$ (3 of 5; 60%) and Ctnnb1$^{del/del}$ (2 of 5; 40%) CMP- Meis-Hoxa9 pre-LSCs developed leukaemia (p = 0.5737) (Figure 4.14C), suggesting that β-catenin is differentially required in the establishment of LSCs from different cellular origins. PCR genotyping of Ctnnb1 status of leukemic cells showed the presence of the floxed or the deleted Ctnnb1 alleles accordingly (Figure 4.4D and 4.14E). Together, these results complement my in vitro observations and demonstrate that β-catenin is necessary for the development of LSC in vivo initiated by Meis-Hoxa9 in LSK but not in CMP populations.

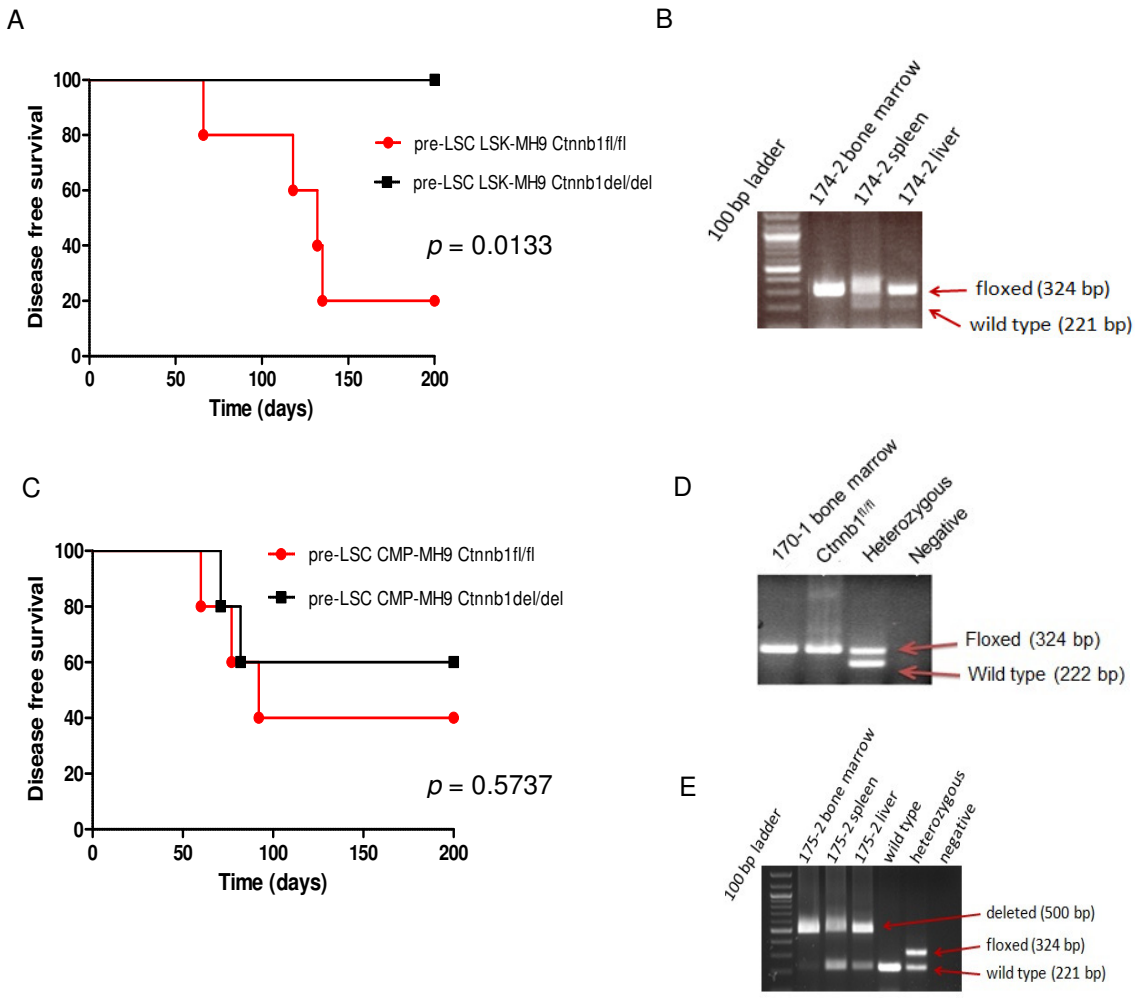


Figure 4.14: LSK but not CMP require β-catenin for the development of Meis1-Hoxa9 LSC. A) and (C) Survival curve of mice transplanted with pre-LSC LSK- and CMP-Meis1-Hxa9 with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ (n = 5 for each group). Genotyping of Ctnnb1 on gDNA extracted from cells of the indicated tissue from leukaemic mice transplanted with pre-LSC LSK-Meis1-Hoxa9 with Ctnnb1$^{fl/fl}$ (B), pre-LSC CMP-Meis1-Hoxa9 with Ctnnb1$^{fl/fl}$ (D) and Ctnnb11$^{del/del}$ (E).

4.3.2 MN1 LSCs originate only from CMPs and do not require β-catenin

To further investigate the potential origins of other LSCs and their β-catenin dependencies, I next tested the MN1 oncogene which is frequently over-expressed in AML. Sorted HSC, CMP, and GMP cells from bone marrow of Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mice were transduced with MN1 retrovirus and subjected to RTTA assay. Genetic deletion of β-catenin was achieved at the early phase of the development of MN1 pre-LSC by adding 20nM tamoxifen in the 2nd round of plating followed by FACS sorting of YFP positive cells. As shown in figure 4.15, knockout of β-catenin did not affect the cloning efficacy and colony morphology of LSK- and CMP-MN1 pre-LSCs in re-plating assay. However, more differentiated GMP progenitor could not be transformed by MN1 oncogene in vitro. Collectively, the results indicated that establishment of LSK- and CMP-MN1 pre-LSCs is β-catenin independent, and GMP may be not the origin of MN1 LSCs.

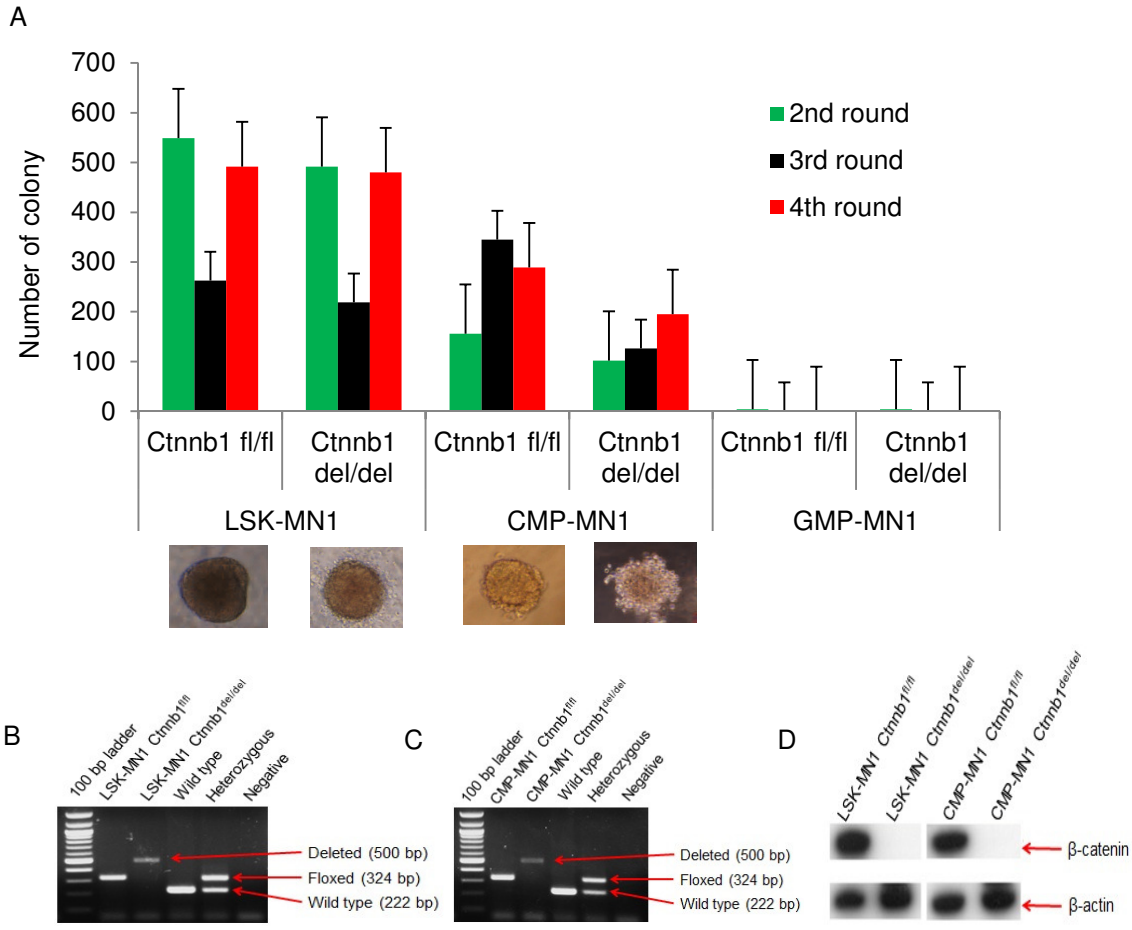


Figure 4.15: GMP not able to transform by MN1 and establishment of LSK- and CMP-MN1 pre-LSC is β-catenin independent. A) Absolute colony numbers (top) and colony morphologies (bottom) of LSK, CMP, and GMP cells transformed by MN1 during replating assay. B) and (C)

Genotyping of Ctnnb1 on gDNA extracted from LSK-MN1 and CMP-MN1 transformed cells after CRE-ER mediated deletion of Ctnnb1 gene by 20nM tamoxifen treatment and YFP sorting. D) Western blot shows a complete deletion of β-catenin in LSK-MN1 and CMP-MN1 transformed cells.

To further investigate the origin of MN1 LSCs and their β-catenin dependency in vivo, LSK-MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ CMP-MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ or with a complete deletion of β-catenin were transplanted into recipient mice (n = 5 for each group). Interestingly, mice which received LSK-MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ had no sign of leukaemia (cut off = 80 days), suggesting that LSK may not be the origin of MN1 LSCs. In contrast, mice that were injected with CMP-MN1 pre-LSCs with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ developed leukaemia with short latency and a median survival of 43 days and 36 days, respectively (figure 4.16). In addition, secondary transplanted mice with LSCs CMP-MN1 with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ developed leukaemia with very short latency (median survival = 14 days for both groups). Together, the results indicated that CMP is the only cell of origin of MN1 leukaemia among all the tested populations, and that initiation of MN1 LSC is β-catenin independent.

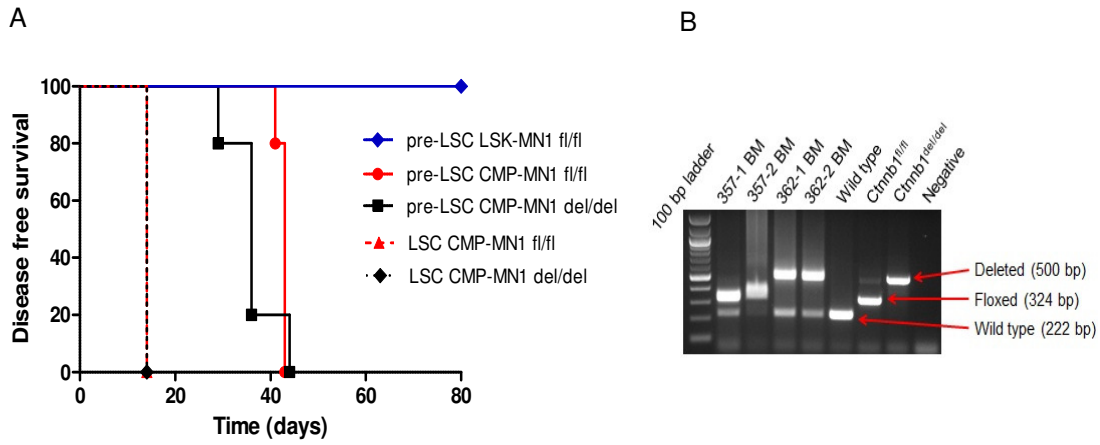
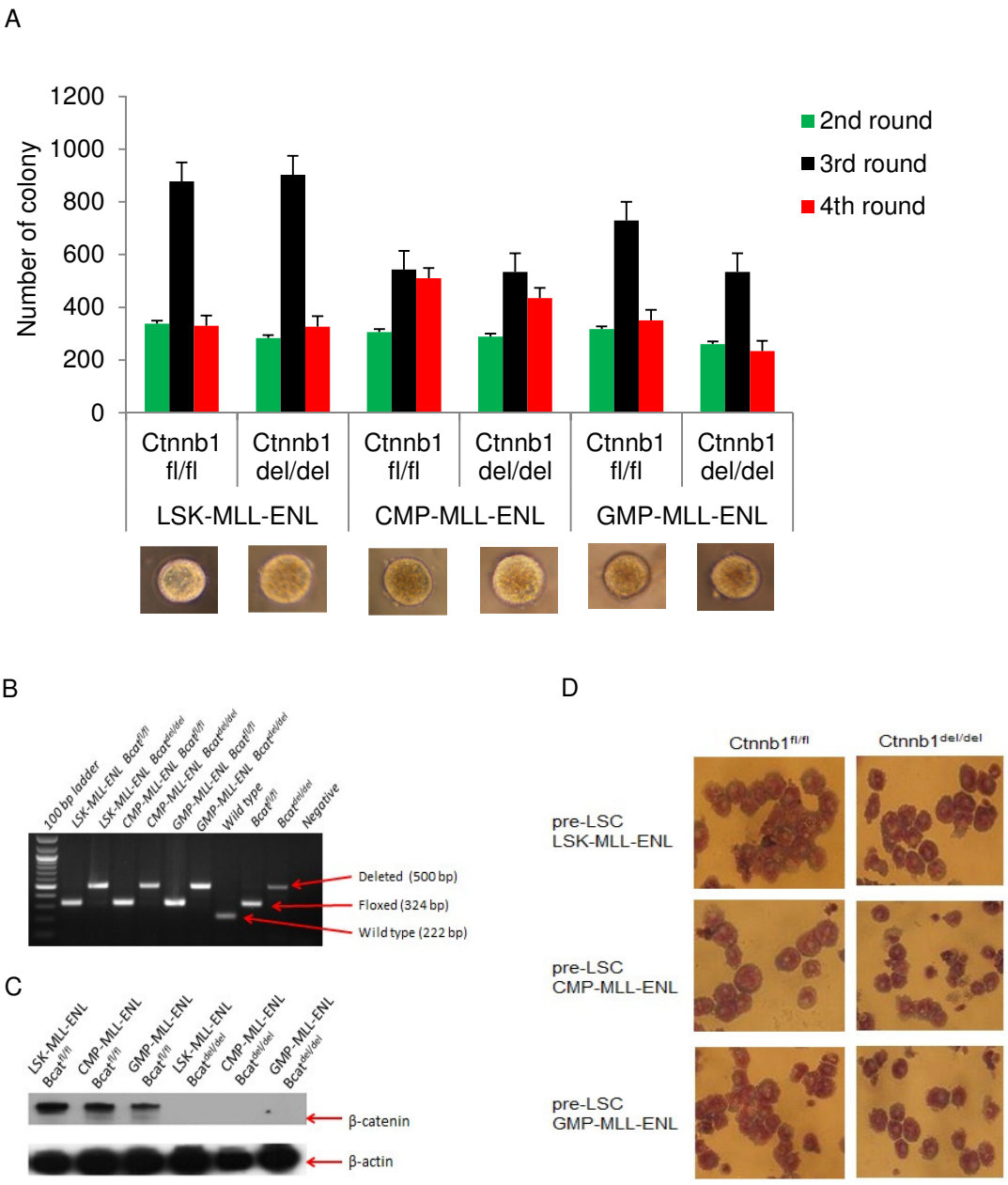


Figure 4.16: CMP-MN1 is β-catenin independent LSC. A) Survival curve of mice transplanted with pre-LSCs LSK-MN1 with Ctnnb1$^{fl/fl}$, CMP-MN1 with Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ (n=5 for each group), and LSC CMP-MN1 with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ (n=5 for each group). B) Genotyping of Ctnnb1 on gDNA extracted from bone marrow of leukaemic mice that received pre-LSC CMP-MN1 with Ctnnb1$^{fl/fl}$ (357-1 and 357-2) and Ctnnb1$^{del/del}$ (362-1 and 362-2).

4.3.3 GMP but not LSK and CMP required β-catenin for the initiation of MLL-ENL LSCs

Genetic deletion of β-catenin has been previous shown by our group to result in eradication of the in vivo oncogenic potential of haematopoietic stem/progenitor cell derived c-Kit+ MLL-ENL pre-LSC (Yeung et al. 2010). To extend this finding, I further investigated whether β-catenin is critical for the development of MLL-ENL LSCs originated from different functionally distinct haematopoietic populations (HSC, CMP, and GMP). HSC, CMP, and GMP cells of Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mice were transduced with retrovirus carrying MLL-ENL (pMSCV-MLL-ENL-puromycin) and subjected to RTTA during which tamoxifen-mediated β-catenin deletion was carried out in the early stage of pre-LSC development. MLL-ENL transformed LSK, CMP, and GMP regardless of β-catenin status with no apparent difference in cloning efficiency, colony and cell morphology as well as immunophenotypes (figure 4.17).



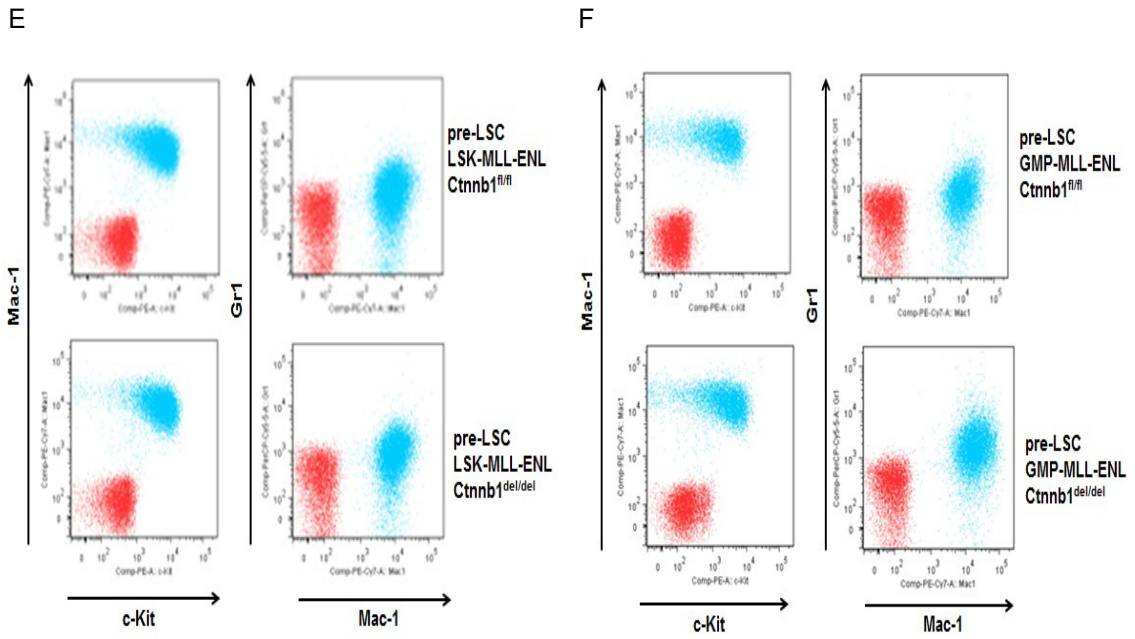


Figure 4.17: β-catenin is not required for the establishment of LSK-, CMP-, and GMP-MLL-ENL pre-LSC. A) Absolute colony numbers (top) and colony morphologies (bottom) of LSK, CMP, and GMP cells transformed by MLL-ENL during replating assay. B) Genotyping of Ctnnb1 on gDNA extracted from LSK-, CMP- and GMP-MLL-ENL transformed cells after CRE-ER mediated deletion of Ctnnb1 gene by 20nM tamoxifen treatment and YFP sorting. C) Western blot shows a complete deletion of β-catenin in LSK-MLL-ENL and CMP-MLL-ENL transformed cells. D) Typical cell morphologies of LSK, CMP, and GMP cells transformed by MLL-ENL after 3rd round of replating. E) Immunophenotypic analysis using flow cytometry of pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ (top) and Ctnnb1$^{del/del}$ (bottom). F) Immunophenotype of pre-LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (top) and Ctnnb1$^{del/del}$ (bottom).

Interestingly, knockout of β-catenin resulted in a slight reduction of dividing cells (G2/M) in pre-LSC GMP-MLL-ENL, whereas largely undisturbed G0/G1, S, and G2/M phases were observed in pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ and complete deletion of β-catenin, suggesting that conditional deletion of β-catenin may only have minor impact on the cell cycle in pre-LSC GMP-MLL-ENL but not pre-LSC-LSK-MLL-ENL in vitro (figure 4.18).

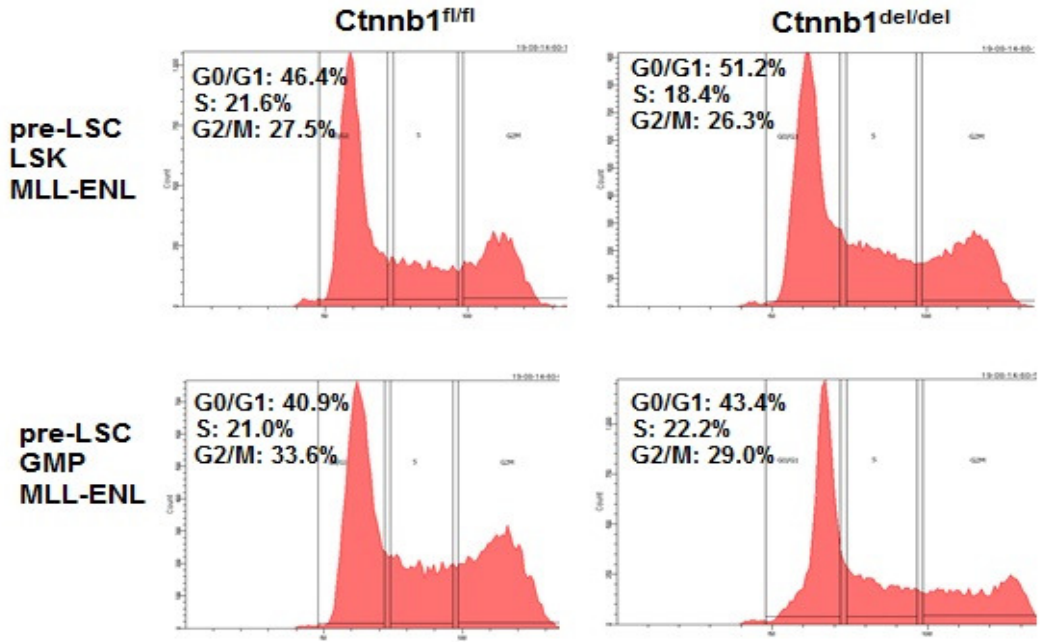


Figure 4.18: Cell cycle analysis of LSK and GMP transformed cells with indicated β-catenin genomic status

To further investigate whether β-catenin is required for the in vivo establishment of MLL LSCs originated from certain cellular origins, MLL-ENL pre-LSC were serial transplanted into irradiated recipient mice. Both LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ and LSK-MLL-ENL with Ctnnb1$^{del/del}$ could induce leukemia in primary recipient animals (8 of 10 mice (80%) median survival = 76.6 days, and 3 of 5 mice (60%) median survival = 112.3 days, p = 0.1276, respectively) (figure 4.19), suggesting that genetic deletion of β-catenin, albeit delaying the disease onset, did not abolish establishment of MLL LSCs. Moreover, secondary recipient mice transplanted with LSC LSK-MLL-ENL with Ctnnb1$^{del/del}$ developed leukaemia with slightly shorten latency (median survival = 20 days) than mice that injected with LSC LSK-MLL-ENL Ctnnb1$^{fl/fl}$ (median survival = 35.6 days), further suggesting that genetic deletion of β-catenin does not affect LSK-MLL-ENL LSCs. Together, these results demonstrated that β-catenin is not required for development of HSC enriched LSK derived MLL-ENL LSC.

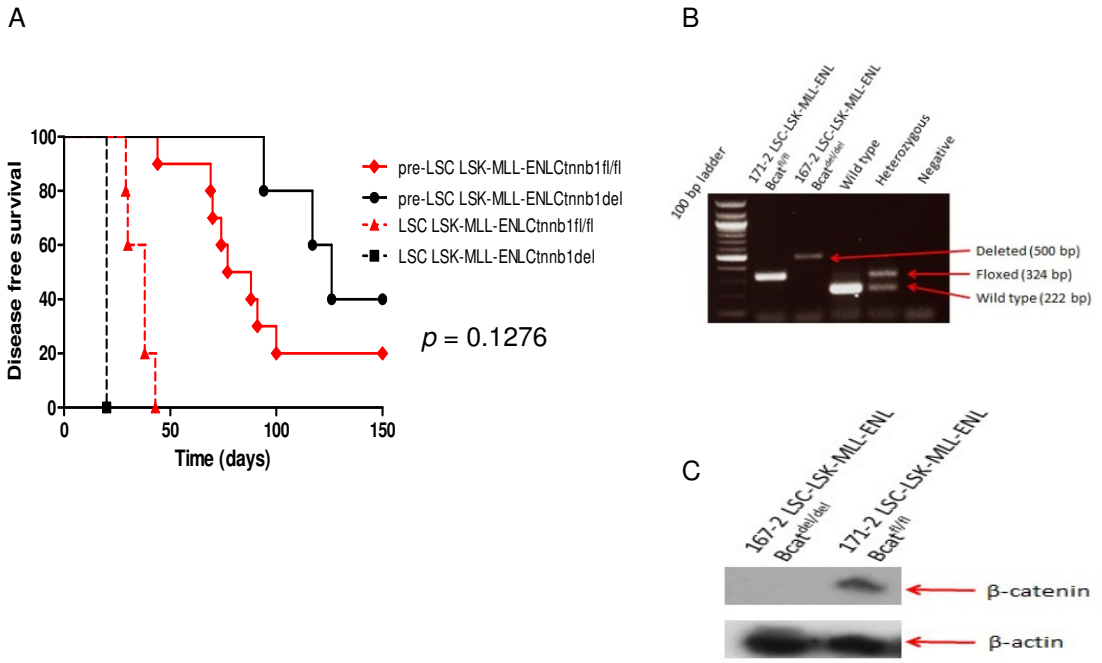


Figure 4.19: LSK-MLL-ENL is β-catenin independent LSC. A) Survival curve of mice transplanted with pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ (n=10), Ctnnb1$^{del/del}$ (n=5), LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ (n=5 for each group). B) Genotyping of Ctnnb1 on gDNA extracted from bone marrow isolated from leukaemia mice transplanted with pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$. C) Western blot shows a complete deletion of β-catenin in LSC-LSK-MLL-ENL.

Similar to LSK, 4 of 5 mice (80%) that received pre-LSC CMP-MLL-ENL Ctnnb1$^{fl/fl}$ developed leukaemia with very short latency (median survival = 55 days). Interestingly, while knockout of β-catenin delayed leukaemia onset of mice that transplanted with pre-LSC CMP-MLL-ENL Ctnnb1$^{del/del}$ (p = 0.0818) (3 of 5 (60%) and median survival = 101 days), no significant difference (p = 0.5356) in disease latency of secondary recipient mice that received both CMP-MLL-ENL LSC with Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ (median survival = 21.8 and 21.2 days, respectively) was observed (figure 4.20). Collectively, these results indicated that MLL-ENL is able to transform and establish LSC originated from committed myeloid progenitor (CMP) and that the development of CMP-MLL-ENL LSC is largely β-catenin independent.

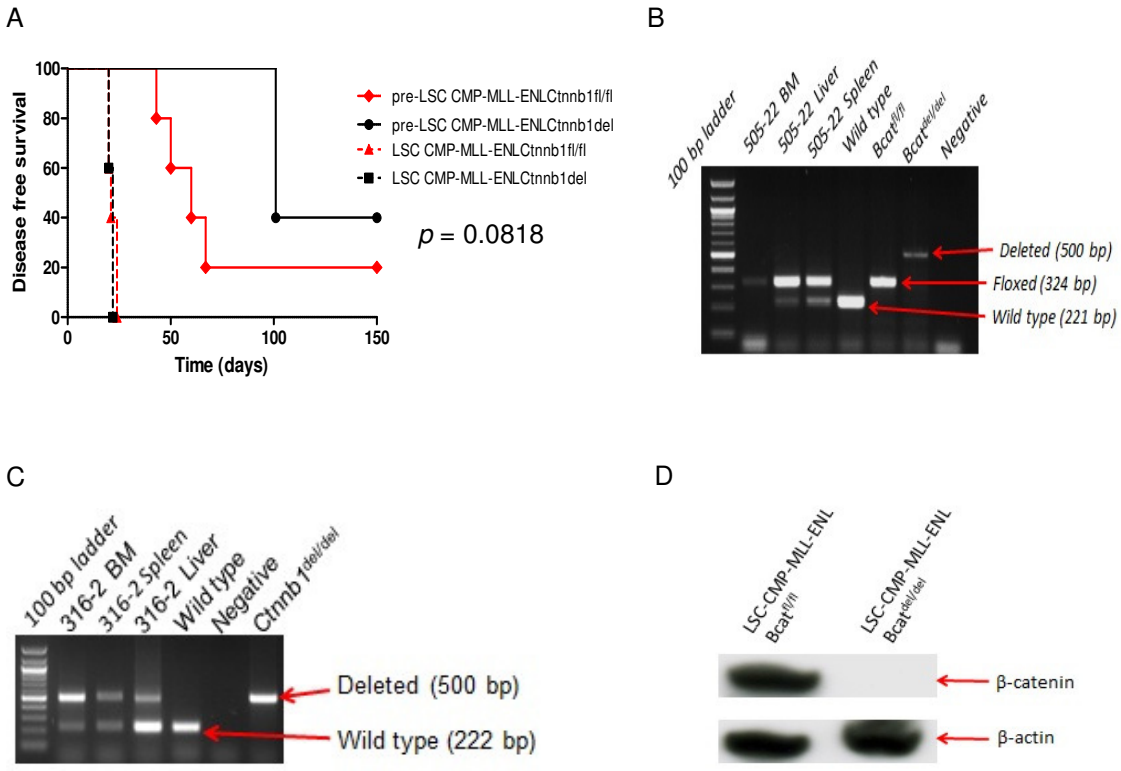


Figure 4.20: CMP-MLL-ENL is β-catenin independent LSC. A) Survival curve of of mice transplanted with pre-LSC CMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (n=10), Ctnnb1$^{del/del}$ (n=5), LSC CMP-MLL-ENL with Ctnnb1$^{fl/fl}$ or Ctnnb1$^{del/del}$ (n=5 for each group). B) and (C) Genotyping of Ctnnb1 on gDNA extracted from bone marrow isolated from leukaemia mice transplanted with pre-LSC CMP-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$. D) Western blot shows a complete deletion of β-catenin in LSC-CMP-MLL-ENL.

In contrast, although 10 of 10 mice (100%) that received GMP-MLL-ENL Ctnnb1$^{fl/fl}$ pre-LSCs developed leukaemia (median survival = 84.6 days) which was transplantable and resulted in much shorter latency in secondary recipient mice (median survival = 19.8 days), mice that were transplanted with MLL-ENL with Ctnnb1$^{del/del}$ pre-LSCs (n = 10) did not show any signs of leukaemia (p < 0.0001) (figure 4.21), consistent with a β-catenin dependent transformation. In summary, my data suggest a contrasting β-catenin requirement for the development of MLL-ENL LSCs based on the cell of origins with LSK- and CMP- derived MLL-ENL LSCs showing β-catenin independency and GMP- derived MLL-ENL LSCs β-catenin dependency. Similar results were observed in four independent experiments (data not show).

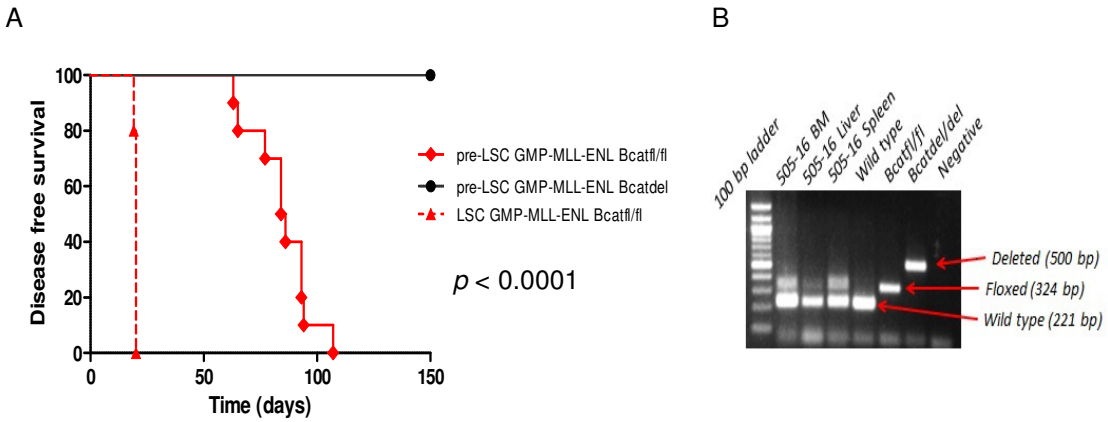
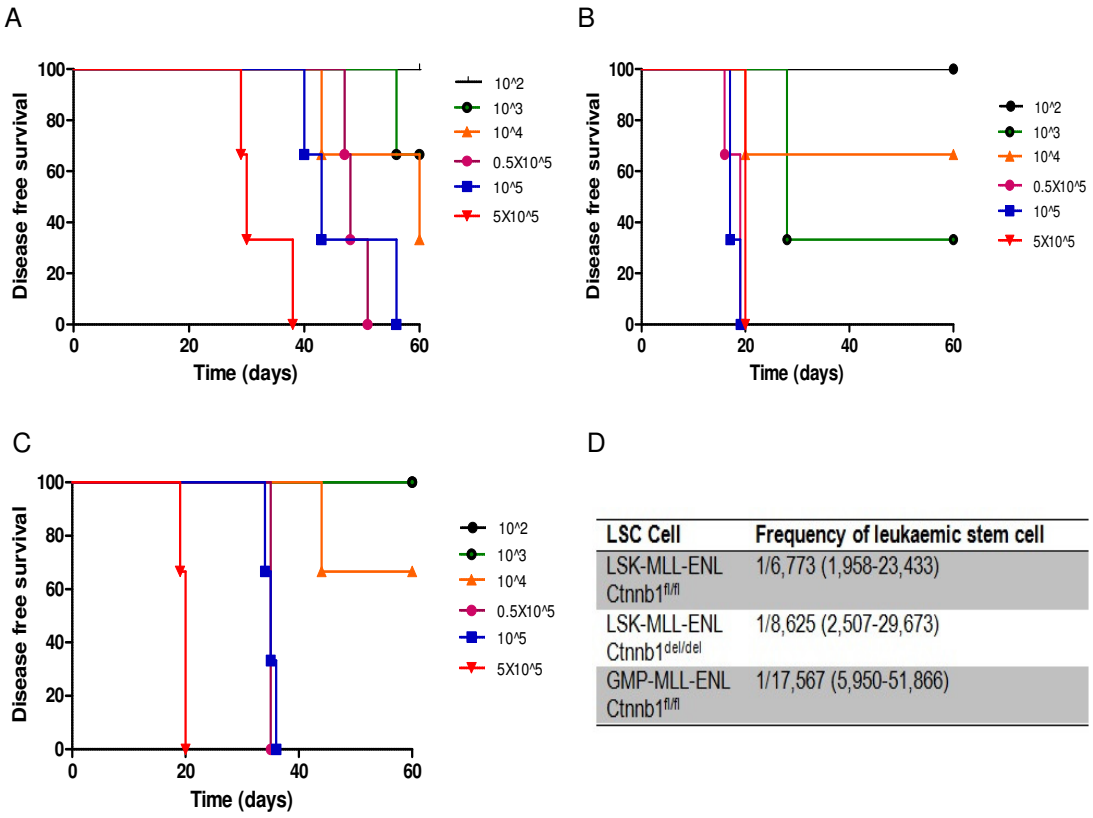


Figure 4.21: β-catenin is critical for the development of GMP-MLL-ENL LSC. A) Survival curve of mice transplanted with pre-LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (n=10), Ctnnb1$^{del/del}$ (n=10), LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (n=5). B) Genotyping of Ctnnb1 on gDNA extracted from bone marrow isolated from leukaemia mice transplanted with pre-LSC GMP-MLL-ENL with bcat$^{fl/fl}$.

To assess the impact of β-catenin deletion on the number of leukaemia initiating cells from MLL-ENL LSCs derived from different cellular origins, I performed the limiting dilution assay (Martínez-Hernández A) by injecting serially increasing numbers of leukemic cells (100, 1,000, 10,000, 50,000, and 100,000 cells for each mouse, n = 3 for each cell number) derived from LSK-MLL-ENL with Ctnnb1$^{fl/fl}$, LSK-MLL-ENL with Ctnnb1$^{del/del}$ and GMP-MLL-ENL with Ctnnb1$^{fl/fl}$. Similar frequencies of leukaemia initiating cells in LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ (1/6,773) and LSK-MLL-ENL with Ctnnb1$^{del/del}$ (1/8,625) were observed, suggesting that deletion of β-catenin has only a minimal impact on the numbers of leukaemia initiating cells within LSK-MLL-ENL LSCs. Interestingly, a slightly lower frequency of leukaemia initiating cells was found in GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (1/17,567) (figure 4.22).



LSC Cell	Frequency of leukaemic stem cell
LSK-MLL-ENL Ctnnb1$^{fl/fl}$	1/6,773 (1,958-23,433)
LSK-MLL-ENL Ctnnb1$^{del/del}$	1/8,625 (2,507-29,673)
GMP-MLL-ENL Ctnnb1$^{fl/fl}$	1/17,567 (5,950-51,866)

Figure 4.22: Frequencies of leukaemia initiating cells of LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$, LSC LSK-MLL-ENL with Ctnnb1$^{del/del}$, and LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$. A), (B), and (C) Survival curve of mice transplanted with different number of LSC cells (100, 1000, 10,000, 50,000, and 100,000 cells for each 3 mice) of LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$, and LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$, respectively. D) Summary of the calculated frequencies of leukaemia initiating cells of each MLL-ENL LSC tested

Together, I could demonstrate a different functional requirement of β-catenin in the development of phenotypically similar MLL-ENL LSC derived from different cellular origins.

Next, I further investigated whether β-catenin is required for the maintenance of GMP derived MLL-ENL LSCs. GMP-MLL-ENL LSC with Ctnnb1$^{fl/fl}$ was conditionally deleted by employing 20nM of tamoxifen into culture medium (R20/20) for 96 hours. Genetic deletion of β-catenin after Cre-ER activation was confirmed by PCR and Western blotting (figure 4.23). 100,000 of LSCs including GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (control untreated cells) and Ctnnb1$^{del/del}$ were transplanted into recipient mice (n = 3 for each group). As a result, all mice that receive GMP-MLL-LSC with Ctnnb1$^{fl/fl}$ developed leukaemia with median survival was 35

days, whereas no sign of disease was observed in mice transplanted with LSC GMP-MLL-ENL with Ctnnb1$^{del/del}$ (p = 0.0246). These results suggest that β-catenin is critical for the maintenance of MLL-ENL LSC originated from GMP progenitor. To confirm those preliminary finding, I currently repeated these experiment by increasing number of recipient mice as well as including the LSK-MLL-ENL LSC to further investigate whether those LSC requires β-catenin for a maintenance of MLL-ENL LSC or not.

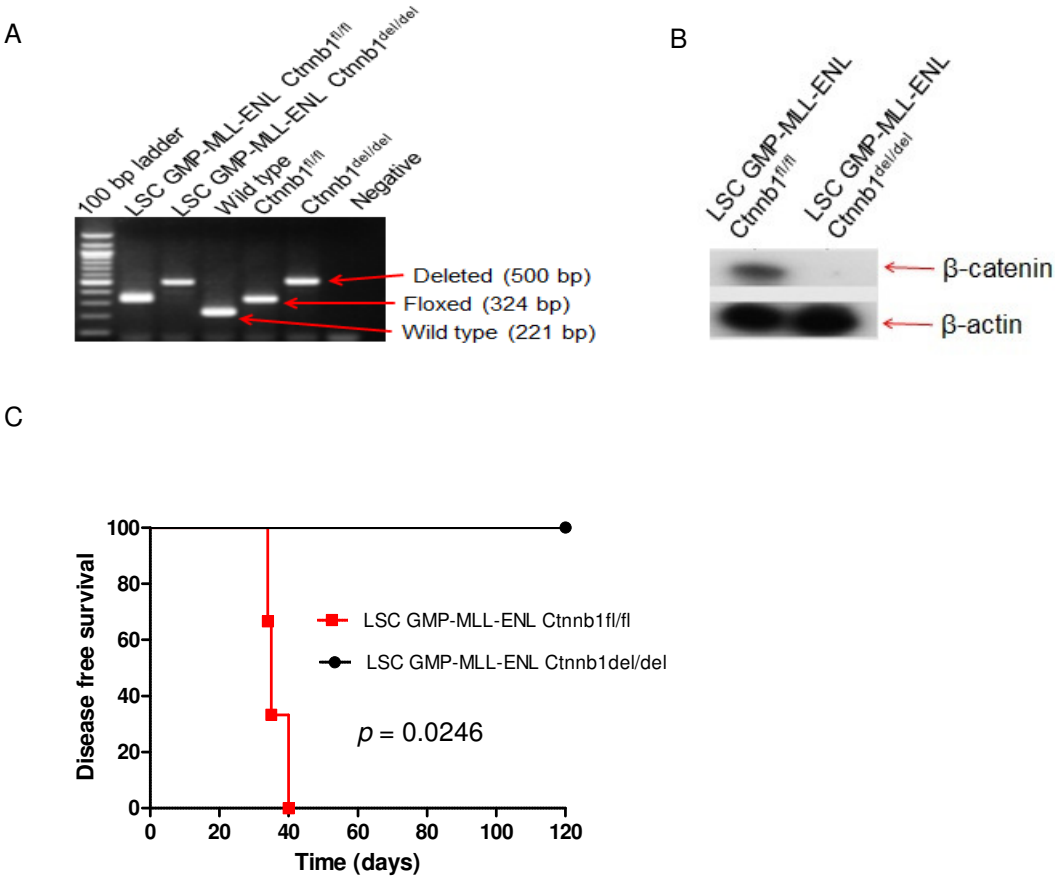


Figure 4.23: β-catenin is required for the maintenance of GMP-MLL-ENL LSC. A) Genotyping on genomic DNA extracted from LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ (control untreated) and LSC GMP-MLL-ENL with Ctnnb1$^{del/del}$ (after incubation with 20nM tamoxifen for 96 hours). B) Western blot shows a complete deletion of β-catenin in LSC GMP-MLL-ENL. C) Survival curve of mice transplanted with LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ after Cre-ER mediated a deletion of β-catenin (n=3 for each group).

4.4 RNA sequencing reveals potential targets of β-catenin in MLL-ENL LSCs derived from different cellular origins

To gain insights into the global mRNA expression and to further investigate the potential targets of β-catenin in MLL-ENL pre-LSCs originated from different origins, I performed RNA sequencing on pre-LSCs including pre-LSC LSK-MLL-ENL and pre-LSC GMP-MLL-ENL with both $Ctnnb1^{fl/fl}$ and complete deletion of β-catenin. As summarised in Table 4.1, two biological replicas of pre-LSC LSK-MLL-ENL with $Ctnnb1^{fl/fl}$ and $Ctnnb1^{del/del}$ samples showed similarities in the number of alignments across the mouse genome as well as a total read (pair end) with mean of alignment = 77.6% and mean of total read = 21.5M for pre-LSC LSK-MLL-ENL with $Ctnnb1^{fl/fl}$, and mean of alignment = 78.8% and mean of total read = 23.5M for pre-LSC LSK-MLL-ENL with $Ctnnb1^{del/del}$, respectively. In contrast, a reduction in number of alignments was observed in both pre-LSC GMP-MLL-ENL with $Ctnnb1^{fl/fl}$ and $Ctnnb1^{del/del}$ samples (4 biological replicas) with mean of alignment = 69.7% and mean of total read = 26M for pre-LSC GMP-MLL-ENL with $Ctnnb1^{fl/fl}$, and mean of alignment = 68.9% and mean of total read = 23.7M for pre-LSC LSK-MLL-ENL with $Ctnnb1^{del/del}$, respectively (Table 4.1).

Table 4.1: Percentage of alignments to mouse genome and total reads of each pre-LSC sample generated by RNA sequencing.

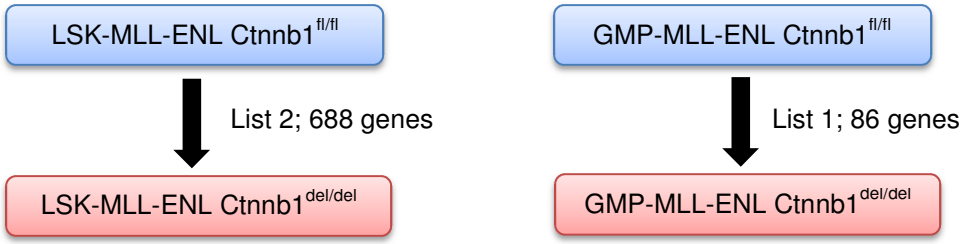
pre-LSC sample	β-catenin status	% of alignment	Total read (pair end)	Experiment
LSK-MLL-ENL	$Ctnnb1^{fl/fl}$	22M	79.40%	
	$Ctnnb1^{del/del}$	27M	81.10%	60.1
LSK-MLL-ENL	$Ctnnb1^{fl/fl}$	21M	75.80%	
	$Ctnnb1^{del/del}$	20M	76.50%	69.1
GMP-MLL-ENL	$Ctnnb1^{fl/fl}$	28M	66.70%	
	$Ctnnb1^{del/del}$	23M	64.90%	51.5
GMP-MLL-ENL	$Ctnnb1^{fl/fl}$	21M	70.40%	
	$Ctnnb1^{del/del}$	21M	67.40%	65.5
GMP-MLL-ENL	$Ctnnb1^{fl/fl}$	26M	68.70%	
	$Ctnnb1^{del/del}$	27M	70.90%	60.5
GMP-MLL-ENL	$Ctnnb1^{fl/fl}$	29M	73.10%	
	$Ctnnb1^{del/del}$	24M	72.50%	69.5

At the gene level using a stringent criteria (p-value < 0.05 and fold change > 2.0), I could identify 688 genes differentially expressed in pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ compared to pre-LSC LSK-MLL-ENL with Ctnnb1$^{del/del}$ using DSeq2. On the other hand, 86 genes were differentially expressed in pre-LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ compared to pre-LSC GMP-MLL-ENL with complete deletion of β-catenin using Limma. Using Vennplex software (Cai H 2013) to analyse two different gene expression datasets (figure 4.24), I found that 408 genes were specifically up-regulated in pre-LSC LSK-MLL-ENL in the absence of β-catenin. Among those, several genes have been reported in many types of cancers as well as in leukaemia such as *Ccl2* (Burgess M 2012) ($p < 0.001$, log2 fold change = 2.85), as *Ccl4* (Yan XJ 2011) ($p < 0.001$, log2 fold change = 3.18) (Burgess M 2012), *Cxcl1* (Hatfield KJ 2010) ($p < 0.001$, log2 fold change = 2.43), *Cxcl2* (Burgess M 2012) ($p < 0.001$, log2 fold change = 2.52), *Cxcl3* [(Mahadevan D 2009)] ($p < 0.001$, log2 fold change = 3.13), *Maf* (Martínez-Hernández A 2014) ($p < 0.001$, log2 fold change = 2.85), *Mmp8* (Kim Y 2014) ($p < 0.001$, log2 fold change = 1.15), Mmp9 (Jiang L 2012) ($p < 0.001$, log2 fold change = 1.74), *Pla1a* (Paulo P 2012) ($p < 0.001$, log2 fold change = 3.00), and *Wnt5b* (Lu D 2004)'(Takeshita A 2014) ($p < 0.001$, log2 fold change = 1.82). In contrast, loss of β-catenin resulted in explicit down-regulated of 228 genes, which are presumably β-catenin targets in pre-LSC LSK-MLL-ENL. Those genes included *Tifab* ($p < 0.001$, log2 fold change = 2.35), *Chpf* ($p < 0.001$, log2 fold change = 2.10), *Tgfb2* ($p < 0.001$, log2 fold change = 1.50), *Ptprf* ($p < 0.001$, log2 fold change = 2.01), and *Ifitm1* ($p < 0.001$, log2 fold change = 2.00). In pre-LSC GMP-MLL-ENL, 12 genes were specifically up regulated in the absence of β-catenin including *Fos* ($p < 0.001$, log2 fold change = 1.40), *Mogat2* ($p < 0.001$, log2 fold change = 1.1), *Pros1* ($p = 0.04$, log2 fold change = 1.38), and *Rhod* ($p < 0.04$, log2 fold change = 1.45). Interestingly, knockout of β-catenin in pre-LSC GMP-MLL-ENL resulted in down-regulation of several genes involved in hypoxia as well as myeloid cells differentiation. Those genes included *Stc1* ($p = 0.01$, log2 fold change = 3.22), *Mboat2* ($p = 0.02$, log2 fold change = 2.15) and *Krt19* ($p = 0.04$, log2 fold change = 3.43), and *Vldlr* ($p = 0.02$, log2 fold change = 1.60). Together, RNA sequencing revealed different expression profiles in LSK- and GMP-derived MLL-ENL pre-LSCs and different potential β-catenin downstream targets which may contribute to the different requirements of β-catenin in phenotypically similar MLL-ENL pre-LSCs derived from different cellular of origins (HSC and GMP).

Despite distinctive expression patterns of many genes after β-catenin knockout across origin specific MLL-ENL pre-LSC cells were observed, co-up-regulation and co-down-regulation

of certain genes also occurred when LSK- and GMP-MLL-ENL pre-LSCs with knockout of β-catenin were compared. 21 commonly overexpressed genes were observed; among those were several genes critical for tumorigenesis as well as leukaemogenesis including *Plk2*, *CD80*, *Lyx1*, *Lyz2*, *Camp*, *Olfm4*, *Map6*, *Trib1*, and *Ipcef1*. Furthermore, 31 genes were co-suppressed in these pre-LSCs when β-catenin was deleted. Those could possibly represent β-catenin targets in both LSK- and GMP-MLL-ENL pre-LSC and included *Samd5*, *Tgfbr3*, *Irf4*, *Ambp*, *Aldoc*, *Tgm2*, and *Thbs1* (table 4.2). Expression levels of those genes were confirmed by qRT-PCR on RNA extracted from pre-LSC LSK- and GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ (figure 4.25). In summary, I could identify both origin-specific as well as origin-independent putative targets of β-catenin in MLL-ENL pre-LSCs derived from different cellular origins. .

A



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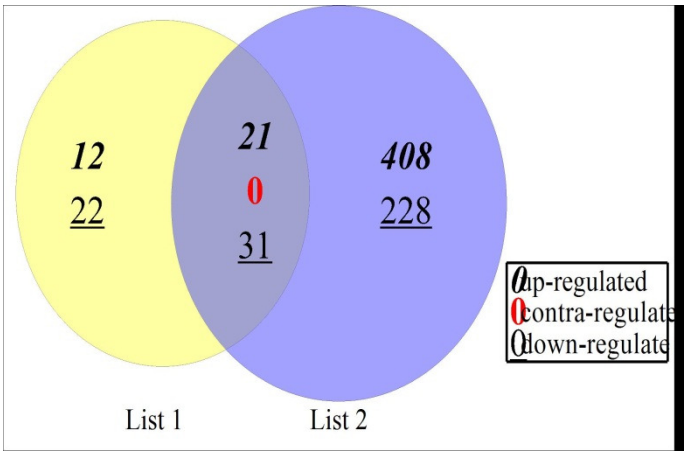


Figure 4.24: Schematic of generation of differential expressed genes which were subjected Venni to generate Venn diagram (p-value < 0.05 and fold change > 2.0). B) Venn diagram shows number of genes that are commonly and differentially expressed in tested samples.

Table 4.2: Summary of differentially and commonly expressed genes in LSK- and GMP-MLL-ENL pre-LSCs with β-catenin knockout.

Genes differentially expressed in pre-LSC LSK-MLL-ENL		Genes differentially expressed in pre-LSC GMP-MLL-ENL		Genes common expressed in pre-LSC LSK-MLL-ENL and GMP-MLL-ENL	
Up regulation (show 22 of 408)	Down Regulation (show 22 of 228)	Up regulation (12 genes)	Down regulation (22 genes)	Up regulation (21 genes)	Down Regulation (show 22 of 31)
Cxcl1	*Tgfb2*	*Mogat2*	*Ccr2*	*Olfm4*	*Samd5*
Tmc3	*Wnt9b*	*Serpinb10*	*C3ar1*	*Clec4a2*	*Tgfbr3*
Cd302	*Wnt10b*	*Ptafr*	*Dpep2*	*Ces1d*	*Bpifc*
Dst	*Tifab*	*Fos*	*Nlrp3*	*Plekhg1*	*Irf4*
Slc11a1	*Chpf*	*Chi3l1*	*H2-Ab1*	*Camp*	*Ambp*
Astn2	*S1pr3*	*Asprv1*	*Stc1*	*Lyz1*	*Aldoc*
Cxcl2	*Neurl1b*	*Zfp819*	*Lrrc32*	*Lyz2*	*Zkscan17*
Il1b	*Ptprf*	*B3galt5*	*Htra3*	*Plk2*	*N4bp3*
Nrp1	*Ifitm1*	*Rhod*	*Dusp4*	*Cd80*	*St6gal1*
Ccl2	*Dlc1*	*Tmcc3*	*Mboat2*	*Col5a1*	*Tgm2*
Maf	*Mamdc2*	*Fosb*	*Vldlr*	*Serpinb2*	*Creb5*
Pla1a	*Pik3ip1*	*Pros1*	*Dlk1*	*Il18*	*Nrbp2*
Cxcl3	*Ndrg2*		*Gm8773*	*Slc6a12*	*Myl10*
Ccl4	*Il1r2*		*Kcnc3*	*Vcam1*	*Lpar1*
Mmp8	*Elfn2*		*Pafah1b3*	*Map6*	*Tex2*
Mmp9	*Slc2a3*		*Mgarp*	*Bst1*	*Ccdc85a*
Mmp14	*Jak3*		*G630025P09Rik*	*Hvcn1*	*Rcor2*
Wnt5b	*Mmp28*		*Krt19*	*Clec4n*	*Thbs1*
Cd38	*Slc52a3*		*Adamtsl5*	*Pdlim4*	*Fgf23*
Cd48	*Map3k1*		*Pgbd1*	*Trib1*	*Atp7b*
Cxcl10	*Cd244*		*Syn1*	*Ipcef1*	*Grin1*
Bax	*Fn1*		*Htr2a*		*Parp16*

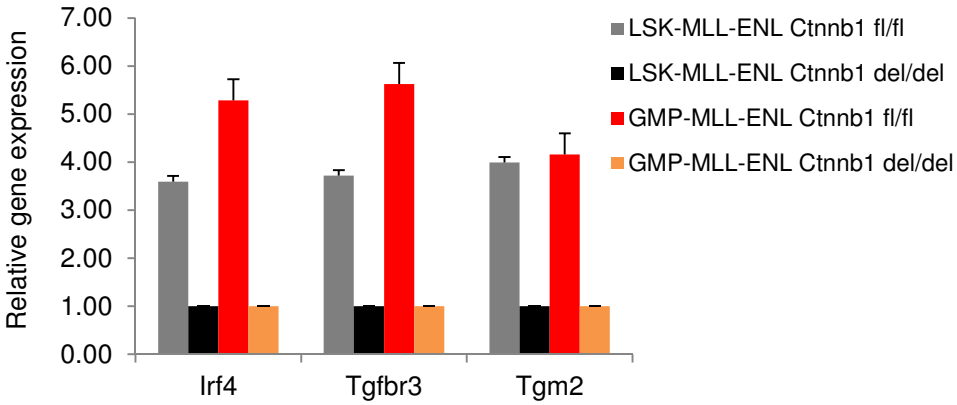


Figure 4.25: Bar chart shows expression levels of downstream targets of β-catenin including *Irf4*, *Tgfbr3*, and *Tgm2* in pre-LSCs LSK-and GMP-MLL-ENL with the indicated genotypes

To further discover the potential pathways being involved in the process, Gene Set Enrichment Analysis (GSEA) was performed with the gene lists described above from the RNA sequencing of the different MLL-ENL pre-LSCs. Using false discovery rate (FDR q Value) less than 0.05, 320 activated- and 141 down-regulated pathways were identified LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ pre-LSCs when compared to LSK-MLL-ENL with Ctnnb1$^{del/del}$ pre-LSCs (figure 4.26). In β-catenin dependent GMP-MLL-ENL pre-LSCs, while 242 pathways were up-regulated, only 7 pathways (myeloid cell development and ion channel transport) were significantly down-regulated in the absence of β-catenin. Furthermore, 117 pathways were co-up- and 5 pathways were co-down-regulated in both LSK- and GMP-derived pre-LSCs upon β-catenin deletion (table 4.3). Collectively, I have globally studied the consequences of β-catenin deletion in MLL-ENL pre-LSCs derived from different cellular origins on the individual gene and pathway levels. Differentially expressed genes and pathways between LSK- and GMP-derived MLL-ENL LSCs in the absence of β-catenin may contribute to the differential biological outcome of β-catenin deletion in these MLL-ENL pre-LSCs.

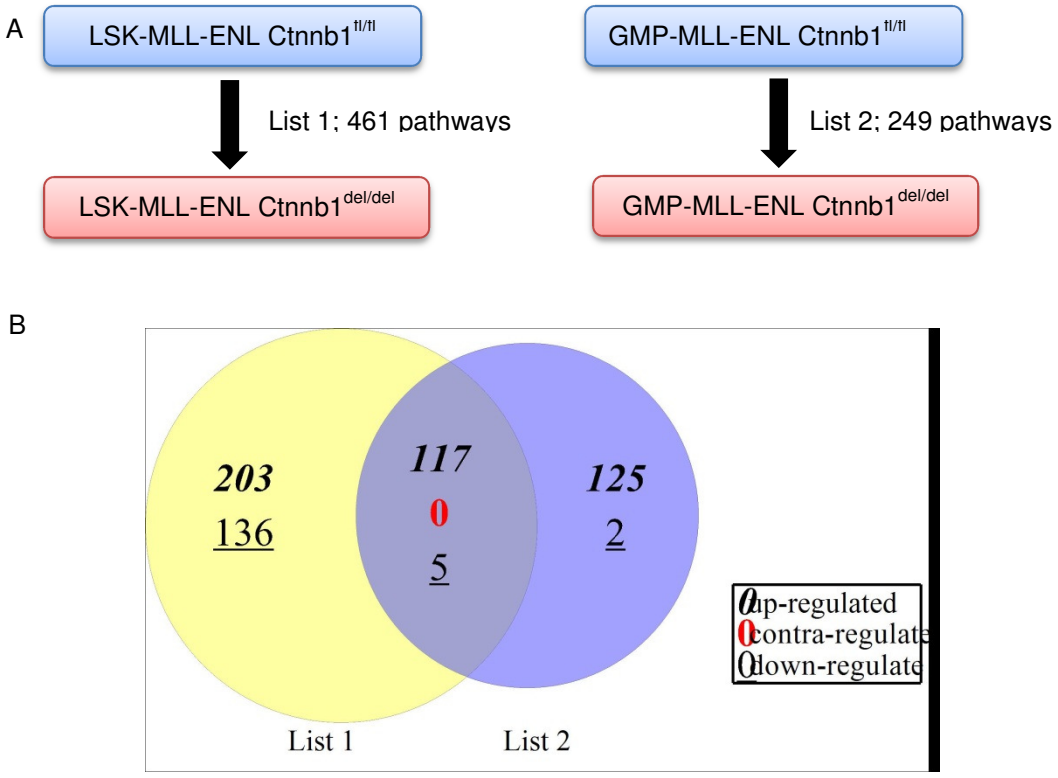


Figure 4.26: Different genes expression Flow chart of input data to generated Venn diagraph (FDR q-value < 0.05). B) Venn diagraph shows number of pathways that common and differentially expressed in tested samples.

Table 4.3: Pathways differential and common expressed in pre-LSC LSK- and GMP-MLL-ENL when conditionally deletion of β-catenin.

Pathway differentially expressed in pre-LSC LSK-MLL-ENL		**Pathway differentially expressed in pre-LSC GMP-MLL-ENL**		**Pathway common expressed in pre-LSC LSK- and GMP-MLL-ENL**	
Up regulation (5 of 203)	**Down regulation (5 of 136)**	**Up regulation (5 of 125)**	**Down regulation (2 pathways)**	**Up regulation (5 of 117)**	**Down regulation (5 pathways)**
REACTOME_PEPTIDE_LIGAND_BINDING_RECEPTORS	CROONQUIST_NRAS_SIGNALING_DN	KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY	BROWN_MYELOID_CELL_DEVELOPMENT_DN	VERHAAK_AML_WITH_NPM1_MUTATED_UP	ZHANG_TLX_TARGETS_60HR_DN
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	LEE_EARLY_T_LYMPHOCYTE_UP	KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	REACTOME_ION_CHANNEL_TRANSPORT	HESS_TARGETS_OF_HOXA9_AND_MEIS1_DN	ZHANG_TLX_TARGETS_36HR_DN
SANA_TNF_SIGNALING_UP	WU_APOPTOSIS_BY_CDKN1A_VIA_TP53	TONKS_TARGETS_OF_RUNX1_RUNX1T1_FUSION_HSC_DN		APPEL_IMATINIB_RESPONSE	FARDIN_HYPOXIA_11
HAN_JNK_SINGALING_UP	ZHAN_MULTIPLE_MYELOMA_PR_UP	YU_MYC_TARGETS_DN		BROWN_MYELOID_CELL_DEVELOPMENT_UP	QI_HYPOXIA
CROONQUIST_NRAS_SIGNALING_UP	REACTOME_SIGNALING_BY_FGFR1_MUTANTS	CHANDRAN_METASTASIS_TOP50_DN		TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_	SEMENZA_HIF1_TARGETS

4.5 Potential molecular interaction between β-catenin and Hox in origin specific MLL-ENL LSCs

Self-renewal pathways are fundamental and essential in normal and/or cancer stem cells, and are down-regulated during differentiation in their downstream progenitors. However, certain molecules including Wnt/β-catenin (table 4.4) can promote, when over-expression, but is not required for self-renewal, suggesting the presence of alternative pathways to compensate for its functions upon genetic deletion. To search for such a pathway, Hoxa9 has been a top candidate that exhibits a similar functional profile as Wnt/β-catenin, which enhances self-renewal but is largely dispensable for normal HSCs. Consistently, I observed that several Hox genes were expressed at a high level in normal HSC and that the expression was reduced throughout differentiation (figure 4.10). Consistently, by quantitative RT-PCR, Hoxa9 was remarkably expressed at a higher level in pre-LSC LSK-MLL-ENL compared to pre-LSC GMP-MLL-ENL (figure 4.27). Interestingly, unpublished data in our group obtained from studying the role of Hoxa9 in origin specific LSCs carried out by former lab members Dr. Jo Sales and Dr. Pedro Veiga, reveal a very similar property of Hoxa9 as compared to β-catenin. It was shown that Hoxa9 was strictly required for the transformation of GMPs by MLL-ENL, whereas it was dispensable for the generation of LSK derived MLL-ENL pre-LSCs and LSCs (figure 4.28). These results are consistent with the RNA-seq data suggesting a possible functional compensation and/or molecular interaction between Hoxa9 and β-catenin only in LSKs, and provide a strong rationale to study a potential functional crosstalk between β-catenin and Hoxa9 in the development of LSK-MLL-ENL LSCs.

Table 4.4; Critical self-renewal pathway and their impact on normal HSC and LSCs; - Negative impact, + positive impact, NS not significant (Zeisig 2012).

Self-renewal pathways		
Loss of function	Impact on	
	HSC	LSC
PTEN	-	+
β-catenin	NS	-
BMI	-	-
HOX	NS	-

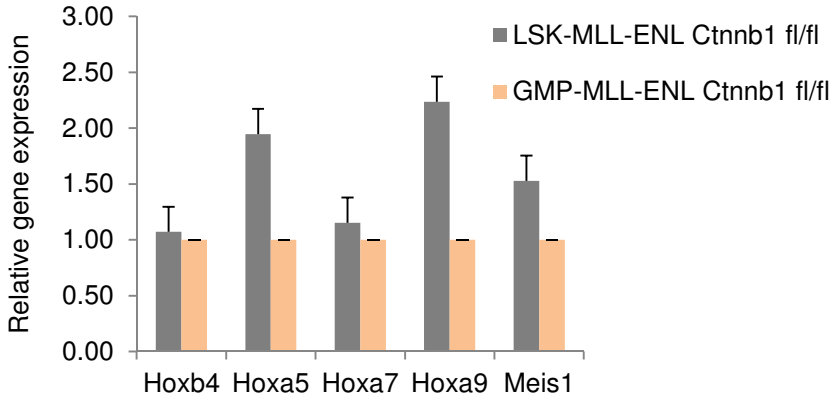


Figure 4.27: Graph shows genes expression by quantitative RT-PCR of *Hoxb4*, *Hoxa5*, *Hoxa7*, *Hoxa9*, and *Meis1* on RNA extracted from LSK- and GMP-MLL-ENL pre-LSC with Ctnnb1$^{fl/fl}$.

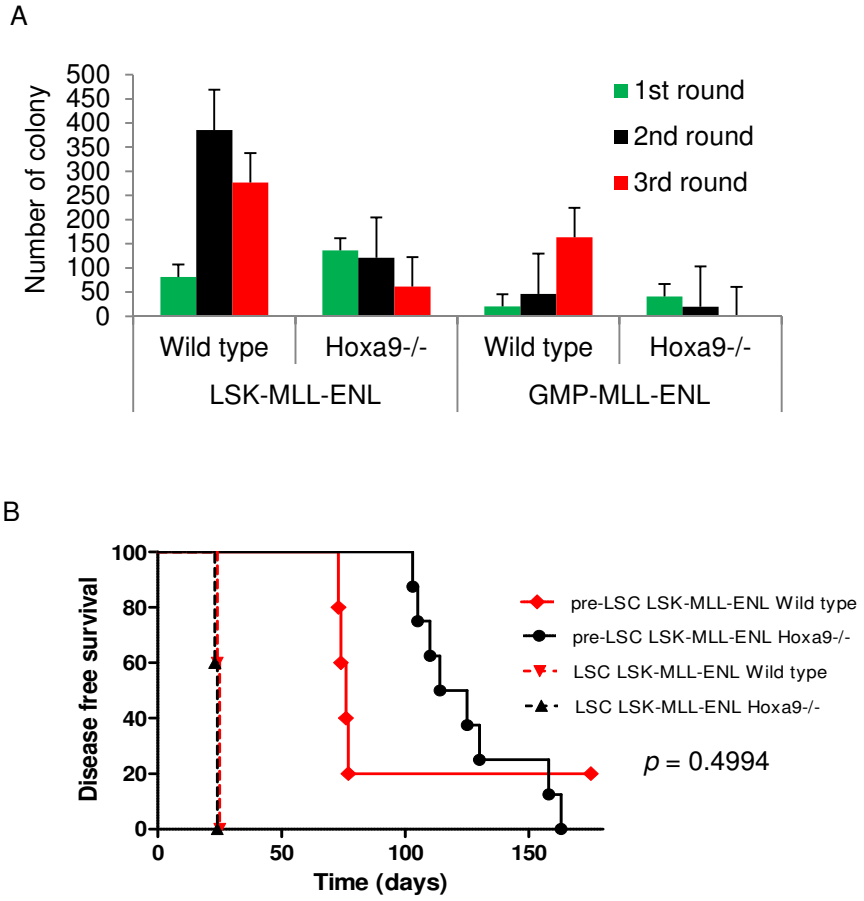


Figure 4.28: GMP but not LSK requires Hoxa9 for the development of LSC transformed by MLL-ENL. A) Bar chart (top) show the absolute number of colonies in each round of plating in MLL-ENL pre-LSCs originated from the indicated cell populations. B) Survival curve of of mice transplanted with pre-LSC LSK-MLL-ENL wild type (n=5), Hoxa9$^{-/-}$ (n=8), LSC LSK-MLL-ENL wild type and Hoxa9$^{-/-}$ (n=5 for each group). Unpublished data from the So Lab generated by Dr. Jo Sales and Dr. Pedro Veiga.

4.6 Compound knockout of β-catenin and Hoxa9 abolish oncogenic potential of MLL-ENL LSCs derived from LSK

To investigate the possible functional crosstalk between β-catenin and Hoxa9 in the development of MLL-ENL LSCs derived from different cellular origins, I have generated a conditionally compound knockout mouse line, Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$/hoxa9$^{-/-}$ in which β-catenin can be inducibly inactivated in Hoxa9 null background. To test if different cellular origins from this new mouse line can be transformed in vitro, I first used E2A-PBX1 transduced cells to RTTA. As seen in figure 4.29, E2A-PBX1 transformed LSK, CMP, and GMP in both wild type and Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ cells, confirming that E2A-PBX1 does not require Hoxa9 in the establishment of pre-LSC in vitro. As expected, MLL-ENL transformed LSK and CMP in the absence of hoxa9 in vitro whereas genetic deletion of Hoxa9 abrogated the transformation ability of MLL-ENL in GMP derived cells (figure 4.30), confirming our previous unpublished data (figure 4.28) that GMP but not LSK requires Hoxa9 in the establishment of MLL-ENL pre-LSC.

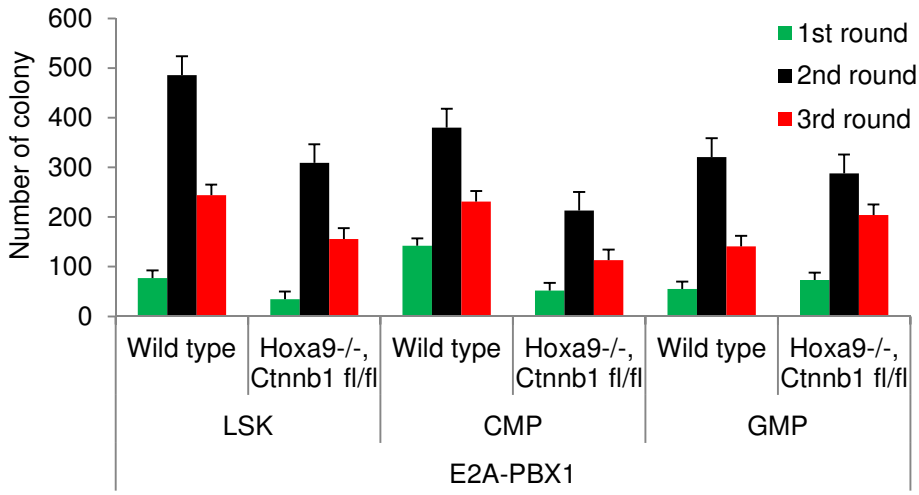


Figure 4.29: Establishment of LSK-, CMP-, and GMP-E2A-PBX1 pre-LSC is independent of Hoxa9. Absolute colony numbers of LSK, CMP, and GMP cells isolated from bone marrow of wild type and Hoxa9$^{-/-}$/Ctnnb1^{flfl} mice transformed by E2A-PBX1 during replating assay.

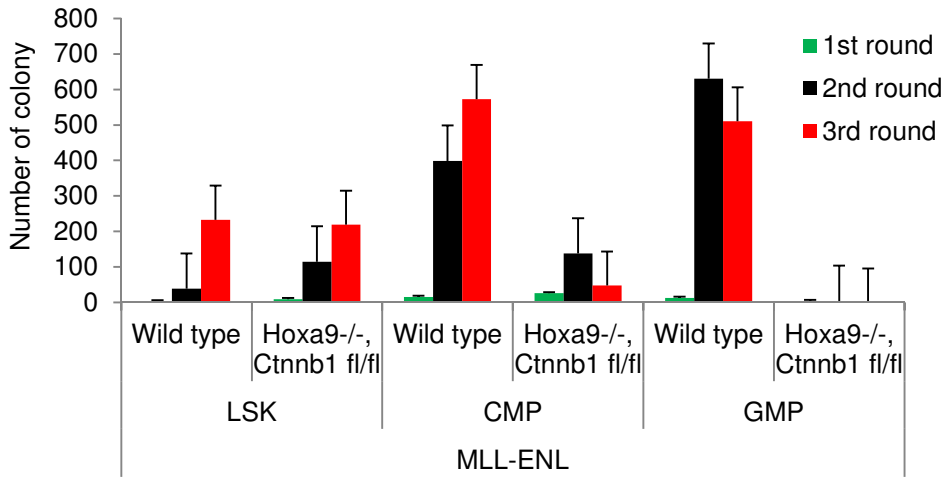


Figure 4.30: Knockout of Hoxa9 suppresses transformation efficacy of CMP-MLL-ENL and GMP-MLL-ENL pre-LSC. Absolute colonies number of LSK, CMP, and GMP cells isolated from bone marrow of wild type and Hoxa9$^{-/-}$/Ctnnb1^{flfl} mice transformed by MLL-ENL during replating assay.

To further investigate the effects of a combined genetic deletion of β-catenin and Hoxa9 on the leukaemogenic potential of Hoxa9 independent pre-LSCs, I performed a conditional deletion of β-catenin in these cells by adding 20nM tamoxifen into the second round of plating. As a result, establishment of LSK- CMP-, and GMP-E2A-PBX1 pre-LSCs were independent on both β-catenin and Hoxa9 in RTTA as they were able to give rise into 4th round colonies regardless of β-catenin and hoxa9 statuses (figure 4.31), demonstrating that compound knockout cells, despite being compromised, can be transformed in vitro.

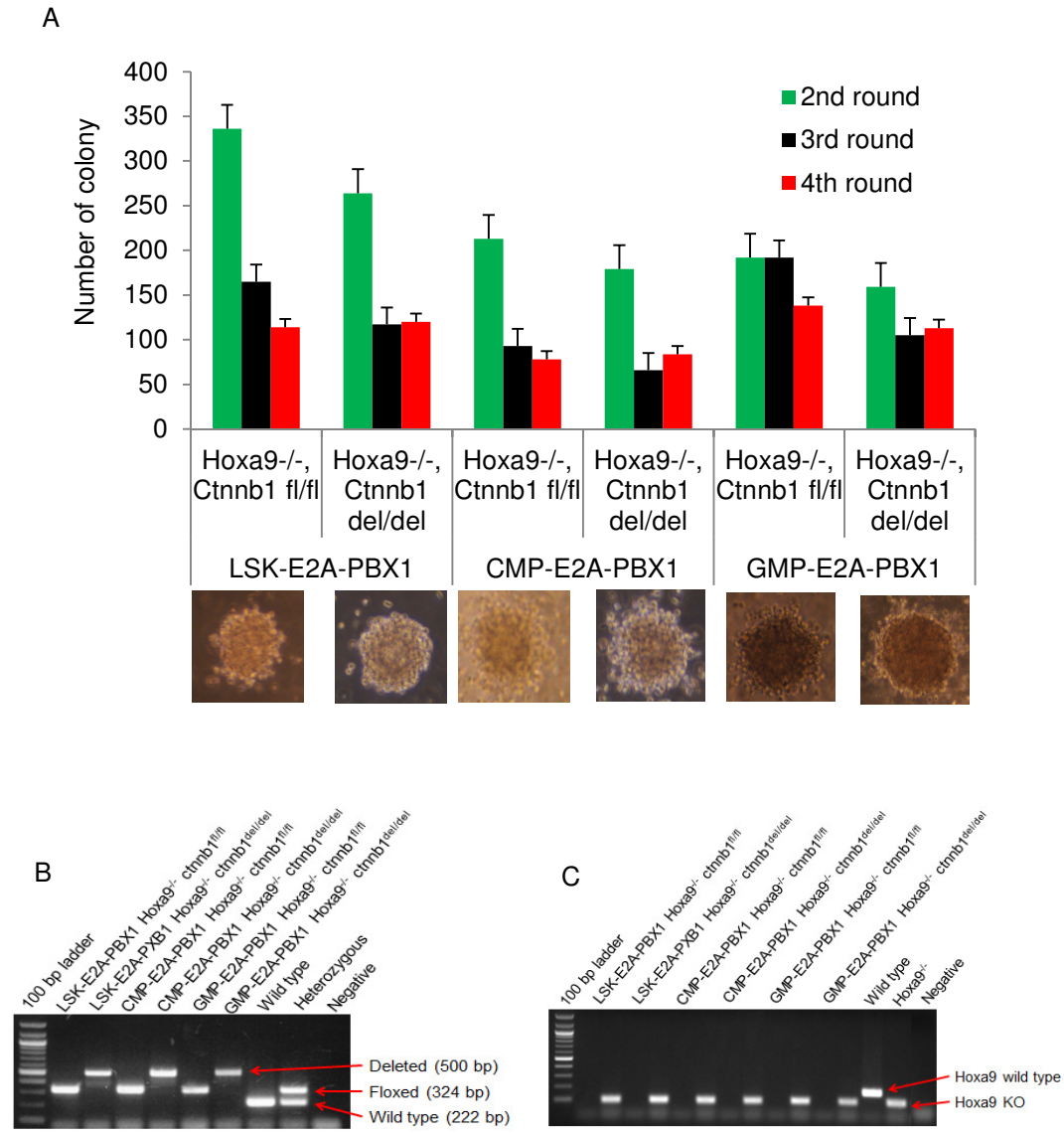


Figure 4.31 Establishment of LSK-, CMP-, GMP-E2A-PBX1 pre-LSC are independent of both β-catenin and Hoxa9. A) Absolute colony numbers (top) and colony morphologies (bottom) of LSK, CMP, and GMP cells transformed by E2A-PBX1 during replating assay. B) and (C) Genotyping of Ctnnb1 and Hoxa9 on gDNA extracted from transformed cells after CRE-ER mediated deletion of Ctnnb1 gene by 20nM tamoxifen treatment and YFP sorting.

Next, I investigated whether compound knockout of β-catenin and Hoxa9 could abrogate transformation efficacy of β-catenin and Hoxa9 independent LSC, the HSC-enriched LSK-MLL-ENL. While MLL-ENL could transform LSK Hoxa9$^{-/-}$ with Ctnnb1$^{fl/fl}$, it failed to transform GMP Hoxa9$^{-/-}$ with Ctnnb1$^{fl/fl}$. Interestingly, compound knockout of β-catenin and Hoxa9 resulted in reduced colony numbers and much-diffused colony morphology in LSK-MLL-ENL cells, suggesting compound knockout of β-catenin and Hoxa9 impaired the transformation

potentials of LSK-MLL-ENL pre-LSCs (figure 4.32). To further investigate and confirm whether compound knockout of β-catenin and Hoxa9 could affect the development of LSK-MLL-ENL LSC in vivo, these pre-LSCs as well as wild type LSK-MLL-ENL pre-LSC were transplanted into recipient mice (n = 5 for each group).

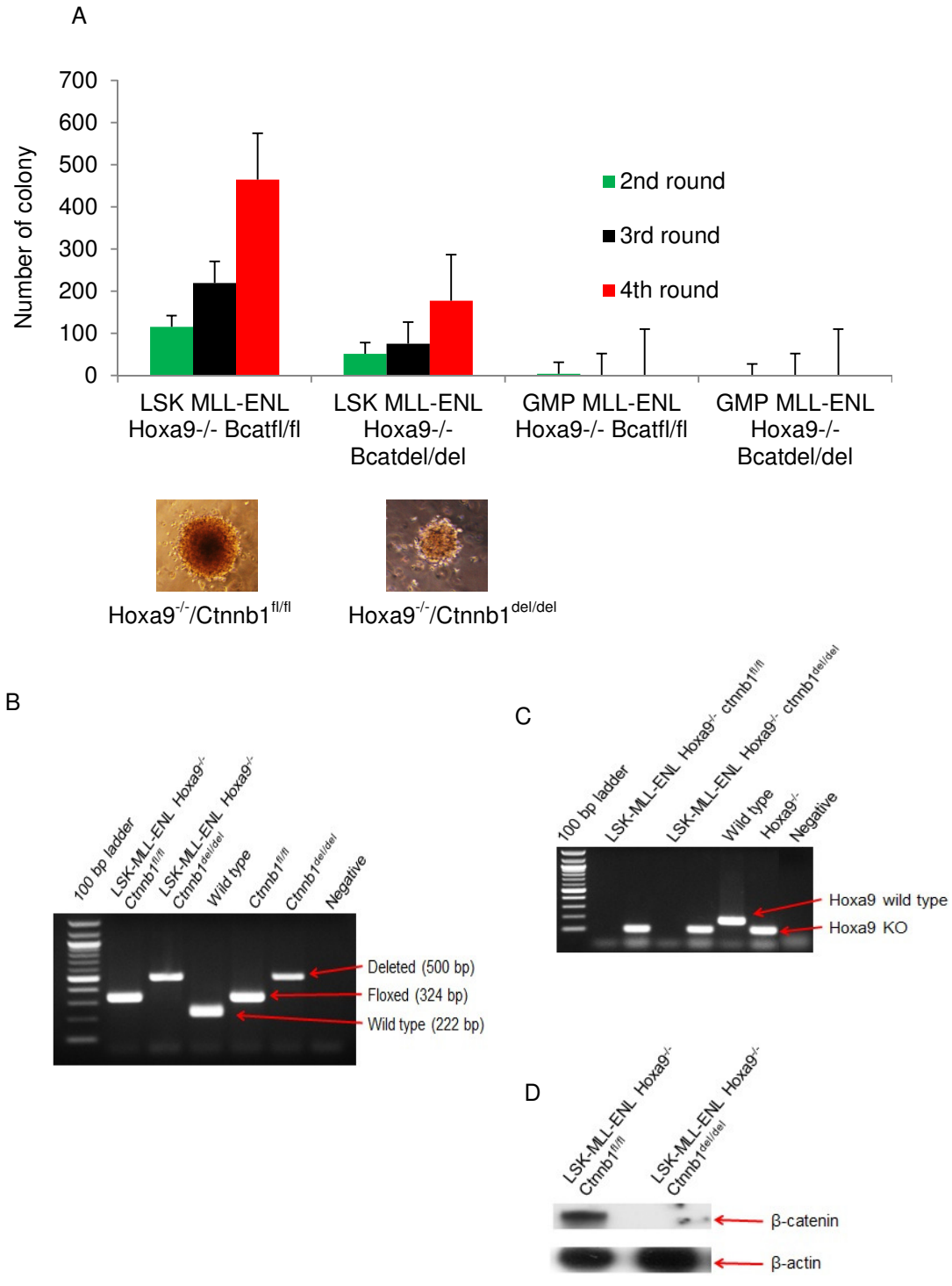


Figure 4.32: Combined genetic deletion of β-catenin and Hoxa9 impairs transformation efficacy of LSK-MLL-ENL pre-LSC in vitro. A) Absolute colonies number and colony morphologies of

LSK, CMP, and GMP cells transformed by MLL-ENL during replating assay. B) and (C) Genotyping of Ctnnb1 and Hoxa9 on gDNA extracted from transformed cells after mediated deletion of Ctnnb1 gene by a treatment of 20 nM tamoxifen and YFP sorting. D) Western blot shows a complete deletion of β-catenin in pre-LSC LSK-MLL-ENL with Hoxa9 knockout.

Consistently with in vitro transformation assay, mice that received wild type LSK-MLL-ENL (4 of 5) and LSK-MLL-ENL Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ (3 of 5) pre-LSCs developed leukaemia with median survival of 75.7 and 54.7 days, respectively. Interestingly, all 5 mice that received compound knockout of β-catenin and Hoxa9 LSK-MLL-ENL pre-LSCs showed no signs of leukaemia (figure 4.33) after 100 days of observation (p = 0.0768). Collectively, these results provide strong evidence of a compensatory mechanism operating in LSKs involving Hoxa9 and β-catenin, and eradicating of MLL LSCs may only be achieved by co-targeting these pathways.

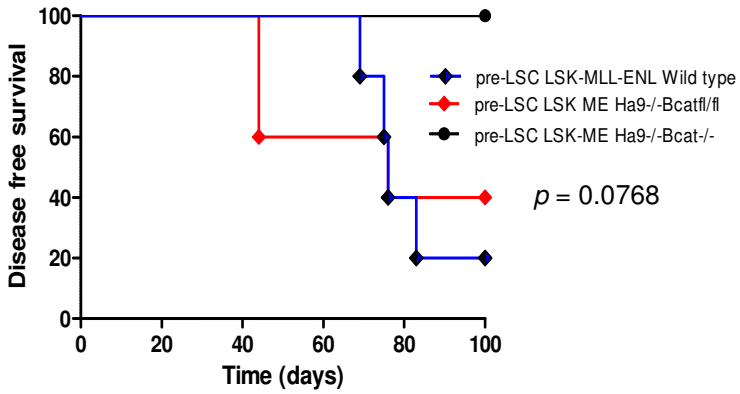
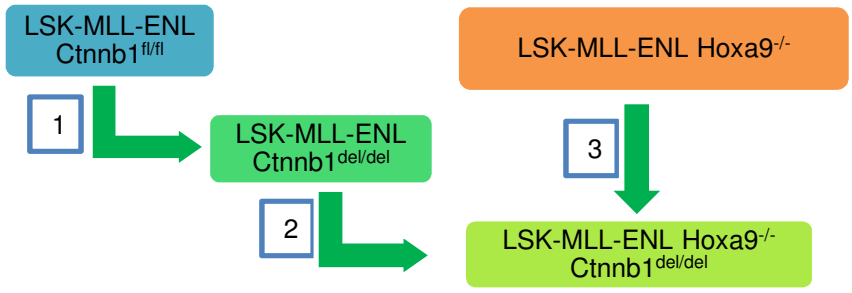


Figure 4.33: Combined genetic deletion of β-catenin and Hoxa9 abolish leukaemogenic potential of pre-LSC LSK-MLL-ENL in vivo. Survival curve of mice transplanted with pre-LSC LSK- MLL-ENL wild type, Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$, and Hoxa9$^{-/-}$/Ctnnb1$^{del/del}$ (n=5 for each group).

4.7 Identification of Prmt1 as a keys modulator to mediate the transformation of MLL-ENL in the absence of β-catenin or Hoxa9

To gain more insights into the relationship between Hoxa9 and β-catenin, RNA sequencing was employed to identify critical molecules that mediate the transformation in one or another's absence. To this end, biological replicas of pre-LSCs, LSK-MLL-ENL with Ctnnb1$^{fl/fl}$, LSK-MLL-ENL with Ctnnb1$^{del/del}$, LSK-MLL-ENL with Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ and Hoxa9$^{-/-}$/Ctnnb1$^{del/del}$ were generated and subjected to RNA sequencing and compared to LSK-MLL-ENL Hoxa9$^{-/-}$ RNA sequencing data already existing in our lab.. Using gene set enrichment analysis (GSEA), I could identify 26 pathways differentially up- and 60 pathways differentially down-regulated in pre-LSC LSK-MLL-ENL with Hoxa9$^{-/-}$/Ctnnb1$^{del/del}$ (figure 4.34), one of which has been the Meis1-Hoxa9 pathway. The following genes, among others within the Meis1-Hoxa9 pathway were significantly down-regulated (p value < 0.05), *Prmt1*, *Ruvbl1*, and *Ruvbl2, Nolc1, and C1qbp*. To confirm the down regulation of these genes, quantitative RT-PCR was performed on pre-LSC LSK-MLL-ENL with both Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$, and Hoxa9$^{-/-}$/Ctnnb1$^{del/del}$ (figure 4.35).

A



B

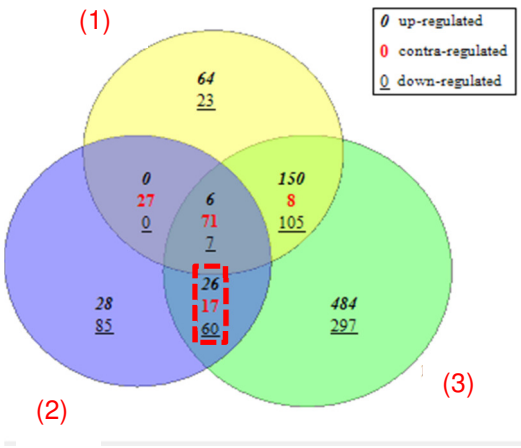


Figure 4.34: GSEA on genes differentially expressed in pre-LSC LSK-MLL-ENL in the absent of β-catenin or Hoxa9 or both of β-catenin and Hoxa9. A) Schematic representation of the different comparisons made after RNA sequencing. Differentially expressed genes were then subjected to GSEA. B) Venn diagraph shows number of pathways that differentially expressed in tested samples.

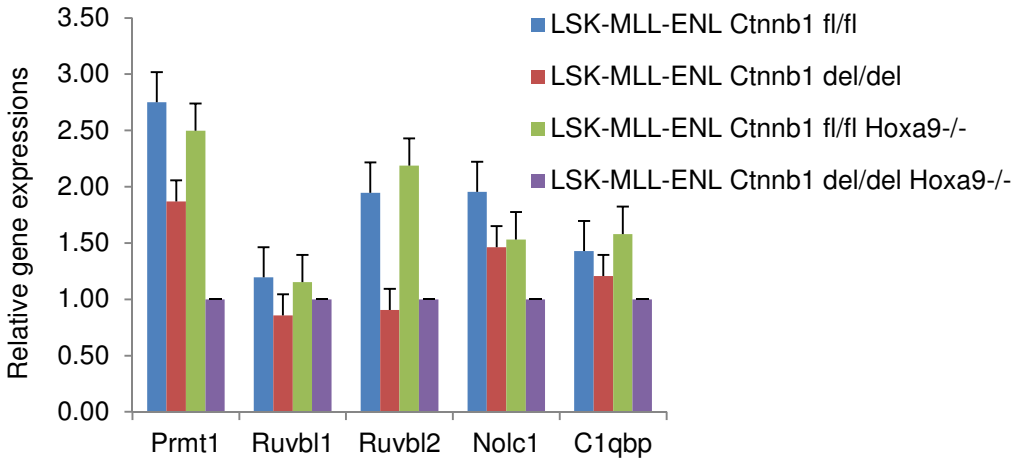


Figure 4.35: Bar chart shows expression levels of genes involved in Meis1-Hoxa9 pathway in pre-LSCs LSK-MLL-ENL with the indicated genotypes.

Among the down regulated genes in Meis1-Hoxa9 pathway, *Prmt1* has been characterised as one of the components in MLL complex and to play an important role in the establishment of LSCs by activating a transcription of several targets including Hox genes (Cheung et al., 2007).To determine if *Prmt1* is a critical mediator for b-catenin/Hoxa9 function in the development of MLL-ENL LSCs, I performed shRNA mediated knockdown experiments targeting *Prmt1* LSK-MLL-ENL Ctnnb1$^{del/del}$ and LSK-MLL-ENL Hoxa9$^{-/-}$ LSCs. After transduction with GFP-tagged PRMT1 shRNA or GFP-tagged vector control, GFP positive cells were sorted and quantitative RT-PCR confirmed great reduction of *Prmt1* mRNA transcripts in both LSC LSK-MLL-ENL Hoxa9$^{-/-}$ and LSC LSK-MLL-ENL Ctnnb1$^{del/del}$ compared to control transduced cells (figure 4.36). Interestingly, when plated in methylcellulose to test for their colony forming abilities, knockdown of *Prmt1* resulted in substantial reduction of colony numbers in LSC LSK-MLL-ENL Hoxa9$^{-/-}$ and LSC LSK-MLL-ENL Ctnnb1$^{del/del}$ (figure 4.37), suggesting that suppression of *Prmt1* could impair the transformation efficacy of LSC LSK-MLL-ENL in both Hoxa9 or β-catenin genetic knockout background.

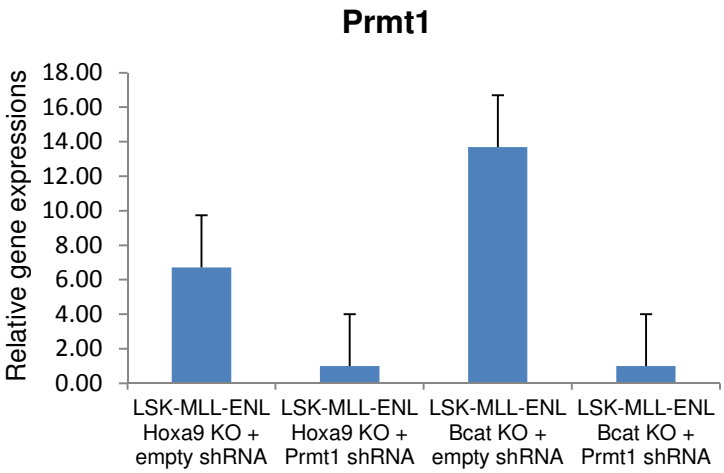


Figure 4.36: Expression of *Prmt1* in LSC LSK-MLL-ENL with the indicated genotypes after transduction with *Prmt1* shRNA or empty vector control.

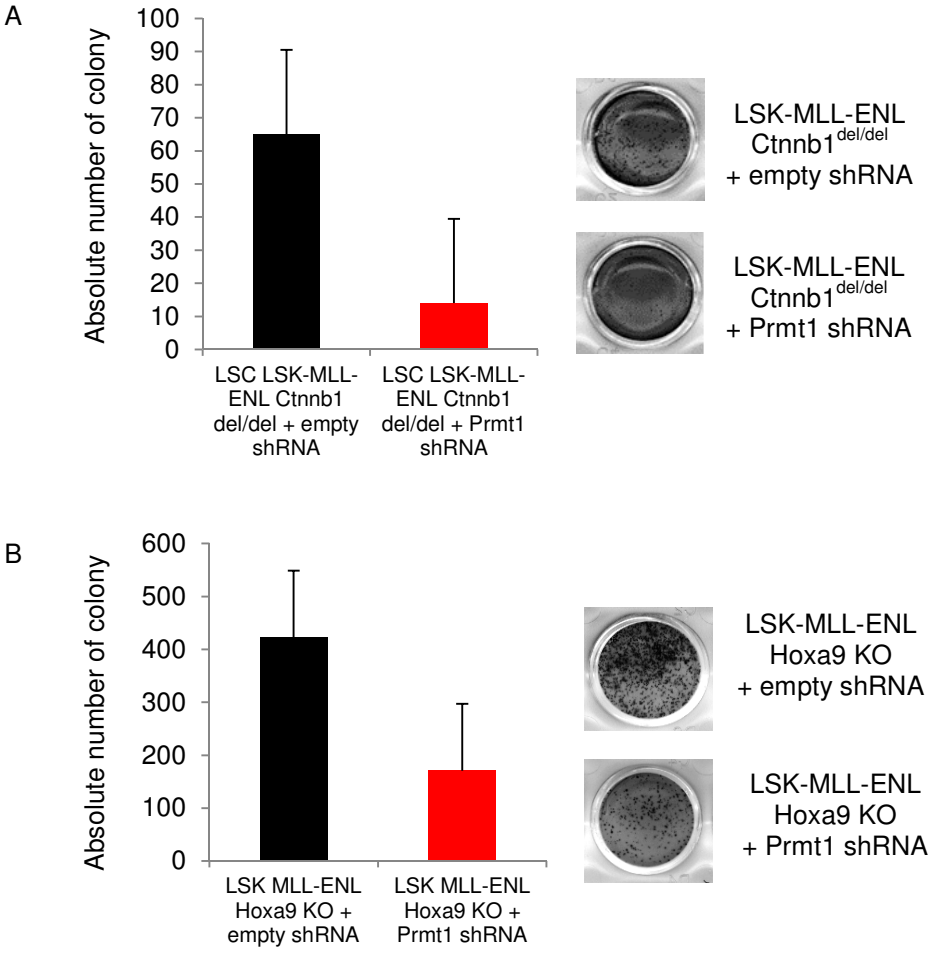


Figure 4.37 Knockdown *Prmt1* suppresses transformation of LSK-MLL-ENL with Hoxa9$^{-/-}$ and LSK-MLL-ENL with Ctnnb1$^{del/del}$ LSCs. Absolute colony numbers and of LSCs LSK-MLL-ENL with Hoxa9 knockout (A) and LSCs LSK-MLL-ENL with knockout of β-catenin (B) after transduced with *Prmt1* and empty control shRNAs.

To further investigate whether suppression of *Prmt1* could also impair the transformation efficacy of those LSC LSK-MLL-ENL in vivo, I transplanted these *Prmt1* knockdown and empty vector control transduced cells to recipient mice (n = 3 for LSK-MLL-ENL Hoxa9$^{-/-}$ transduced with empty vector-GFP, n = 5 for LSK-MLL-ENL Hoxa9$^{-/-}$ transduced with Prmt1-shRNA-GFP, LSK-MLL-ENL Ctnnb1$^{del/del}$ transduced with empty vector-GFP, and LSK-MLL-ENL Ctnnb1$^{del/del}$ transduced with Prmt1-shRNA-GFP). Consistently, all mice (3 of 3) that receive LSC LSK-MLL-ENL Hoxa9$^{-/-}$ transduced with empty vector-GFP developed leukaemia with very short latency (median survival = 25.7 days). In contrast, mice that were transplanted with LSC LSK-MLL-ENL Hoxa9$^{-/-}$ transduced with Prmt1-shRNA-GFP did not show any signs of disease up to day 50 (p = 0.0067) (figure 4.38). These results indicate that genetic deletion of Hoxa9 together with the suppression of *Prmt1* could abolish leukaemogenic property of MLL-ENL LSC derived from HSC-enriched LSK cells. In vivo experiments with *Prmt1* knockdown in LSC LSK-MLL-ENL with β-catenin knockout were just set up and I am now waiting for the first results of these vivo experiments.

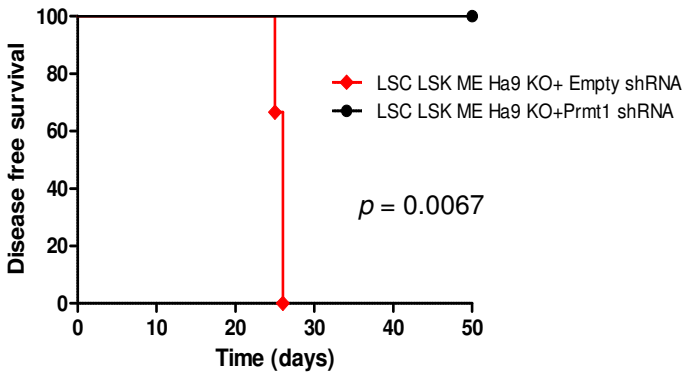
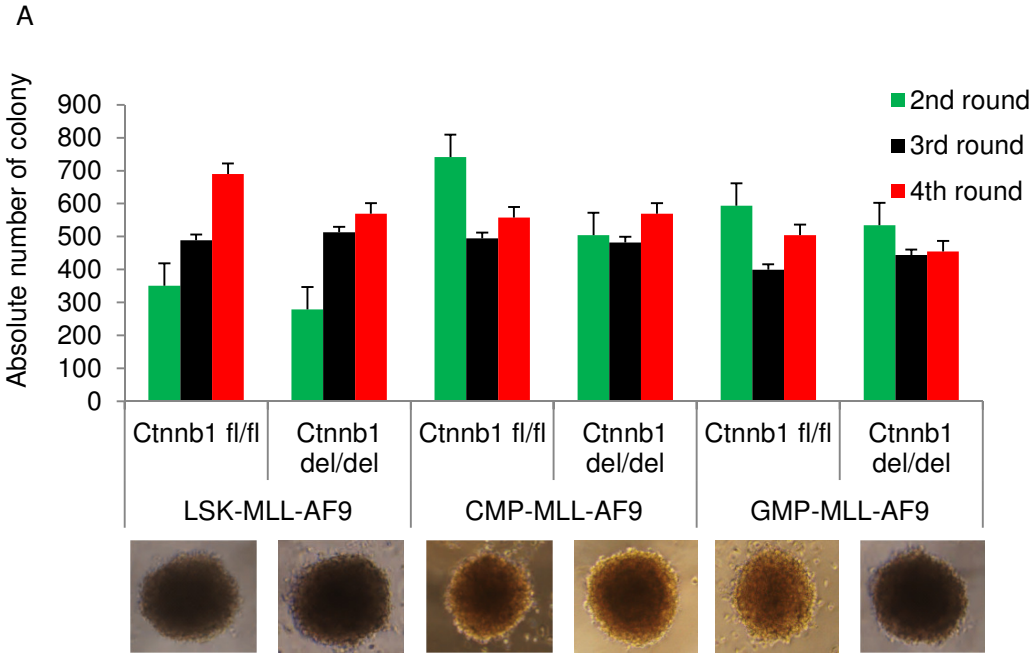


Figure 4.38: Knockdown *Prmt1* impairs leukaemogenic potential of LSK-MLL-ENL LSC with hoxa9$^{-/-}$. Survival curve of mice transplanted with LSC LSK- MLL-ENL with Hoxa9 knockout transduced with Prmt1-shRNA or control empty vector control (n=3 for control and 5 for the test group).

4.8 Transformation of MLL-AF9 LSCs could abolish by combined genetic deletion of β-catenin and Hoxa9

To extend my work into the functional crosstalk between β-catenin and Hoxa9 in the initiation of other AML LSCs, I tested MLL-AF9 as it frequently observed and presented a poor prognosis in AML. Here, I described the impact of genetic deletion of the individual gene, β-catenin and Hoxa9 and a combined genetic deletion of those two molecules in the establishment of MLL-AF9 LSCs which derived from different cell of origins.

Firstly, pools of HSC, CMP, and GMP were purified from Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ mice (5 mice) and subsequently transformed with MLL-AF9. Transformed cells were subjected to RTTA following with genetic deletion of β-catenin at the early phase of the development of MLL-AF9 pre-LSC by adding 20nM tamoxifen into the 2nd round of plating followed by FACS sorting of YFP positive cells. As a result, genetic deletion of β-catenin was not result in a suppression of cloning efficacy of MLL-AF9 in all LSK-, CMP-, and GMP-MLL-AF9 transformed cells (figure 4.39). Thus, establishment of MLL-AF9 pre-LSCs may independent on β-catenin. To further investigate whether β-catenin is required for development of MLL-AF9 LSCs in vivo, LSK, CMP, and GMP pre-LSCs from Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ were transplanted to primary recipient mice (n=5 for each group) for disease development.



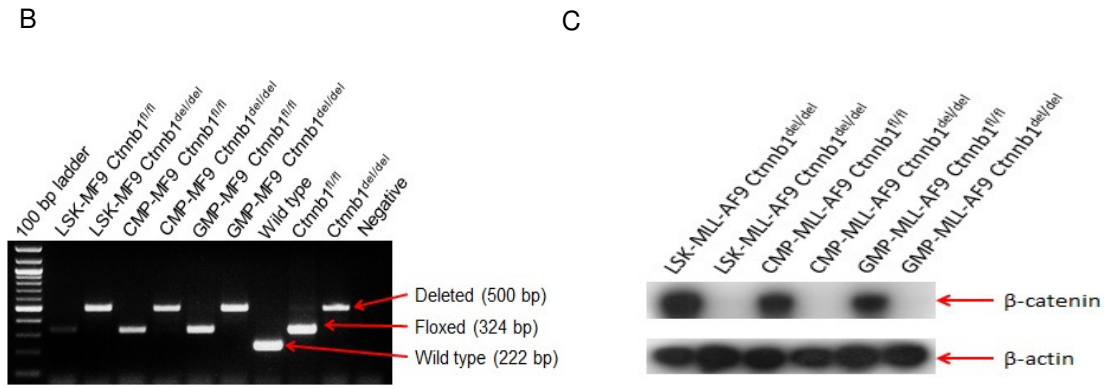


Figure 4.39: β-catenin is not required for the establishment of LSK-, CMP-, and GMP-MLL-ENL pre-LSC. A) Absolute colony numbers (top) and colony morphologies (bottom) of LSK, CMP, and GMP cells transformed by MLL-AF9 during replating assay. B) Genotyping of Ctnnb1 and gDNA extracted from transformed cells after CRE-ER mediated deletion of Ctnnb1 gene by 20nM tamoxifen treatment and YFP sorting. C) Western blot shows a complete deletion of β-catenin in LSK-, CMP, and GMP-MLL-AF9 transformed cells.

In vivo, both LSK-MLL-AF9 with Ctnnb1$^{fl/fl}$ and LSK-MLL-AF9 with Ctnnb1$^{del/del}$ could induce leukemia in primary recipient animals (p = 0.3538) (5 of 5 mice (100%) median survival = 46 days, and 5 of 5 mice (100%) median survival = 41 days, respectively) (figure 4.40A). Similar to LSK-MLL-AF9 LSC, all mice that transplanted with CMP-MLL-AF9 with Ctnnb1$^{fl/fl}$ (n = 5) and CMP-MLL-AF9 with Ctnnb1$^{del/del}$ (n = 5) developed leukaemia (p = 0.9587) with median survival = 40 and 35 days, respectively (figure 4.40B). In contract, while all mice (5 of 5) that received GMP-MLL-AF9 with Ctnnb1$^{fl/fl}$ showed signs of leukaemia with median survival = 58 days, significantly delayed in disease free survival (p value = 0.0020) was observed in mice transplanted with GMP-MLL-AF9 with Ctnnb1$^{del/del}$ (5 of 10 mice, median survival = 113 days) (figure 4.40C). These results indicated that genetic deletion of β-catenin alone was not sufficient to abrogate the transformation of MLL-AF9 LSCs derived from HSC and CMP. However, knockout of β-catenin could impair the leukaemogenic potential of GMP-MLL-AF9 LSC. Collectively, I could demonstrate the different requirement of β-catenin in the development of MLL-AF9 LSCs derived from different cell of origins and eradication of MLL-AF9 LSCs by targeting of β-catenin may largely depend on cell of origins.

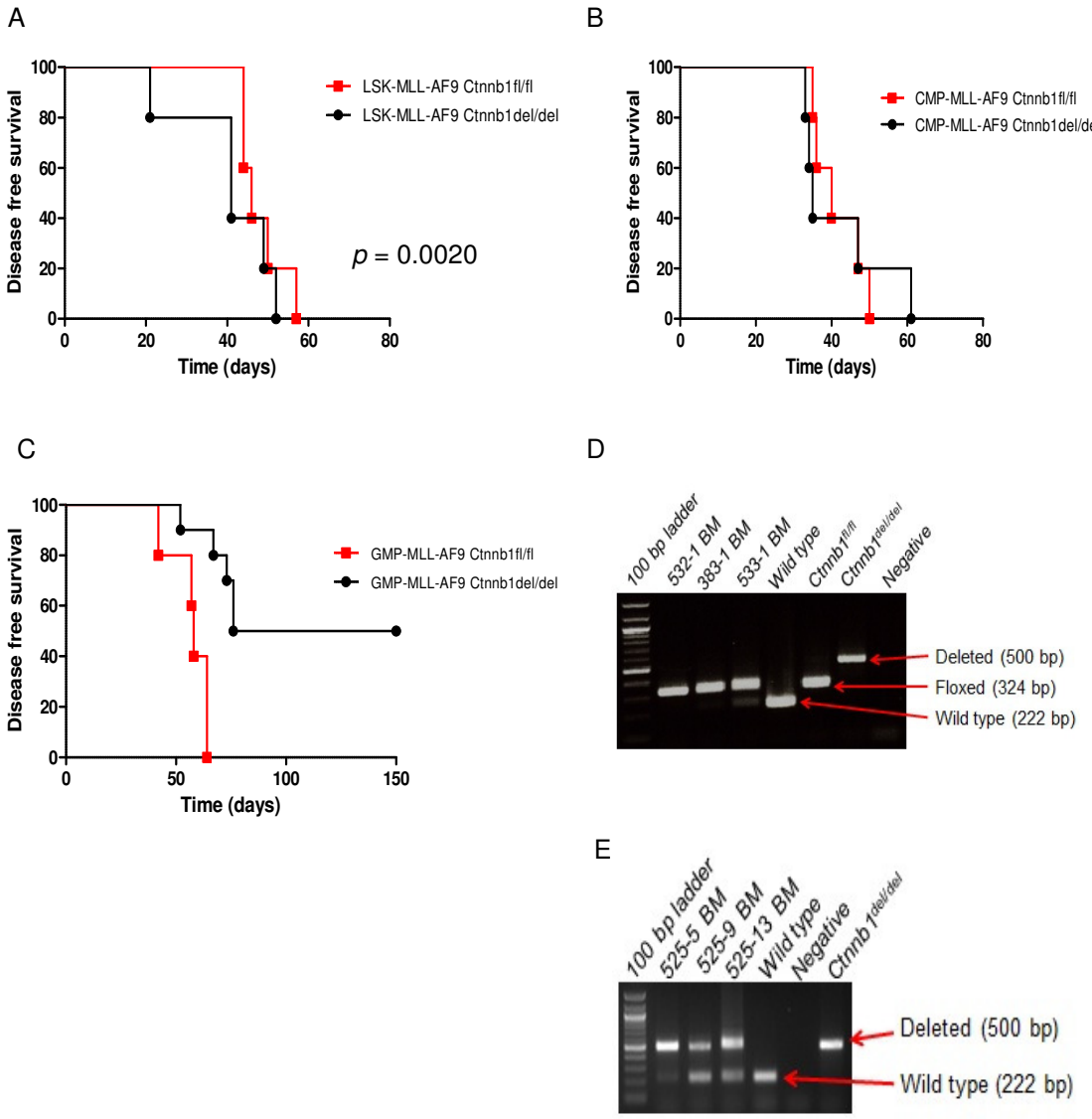


Figure 4.40: Knockout of β-catenin impairs transformation efficacy of pre-LSC GMP-MLL-AF9 but not in pre-LSC LSK-and CMP-MLL-AF9. A) Survival curve of mice transplanted with LSK-MLL-AF9 with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ (n = 5 for each group). B) Survival curve of mice transplanted with CMP-MLL-AF9 with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ (n = 5 for each group). C) Survival curve of mice transplanted with GMP-MLL-AF9 with Ctnnb1$^{fl/fl}$ (n = 10) and Ctnnb1$^{del/del}$ (n = 5). D) and (E) Genotyping of Ctnnb1 on gDNA extracted from bone marrow isolated from leukaemia mice transplanted with pre-LSC LSK- MLL-AF9 with Ctnnb1$^{fl/fl}$ (532-1), CMP-MLL-AF9 with Ctnnb1$^{fl/fl}$ (383-1), GMP-MLL-AF9 with Ctnnb1$^{fl/fl}$ (533-1), and LSK-MLL-AF9 with Ctnnb1$^{del/del}$ (525-5), CMP-MLL-AF9 with Ctnnb1$^{del/del}$ (525-9), GMP-MLL-AF9 with Ctnnb1$^{del/del}$ (525-13), respectively.

Next, I further investigated whether genetic deletion of Hoxa9 alone or combine knockout of β-catenin and Hoxa9 could abolish oncogenic potential of MLL-AF9 LSCs by targeting purified HSC, CMP, and GMP isolated from bone marrow of Rosa26-Cre/Rosa26-YFP/Ctnnb1$^{fl/fl}$ and hoxa9$^{-/-}$ mice. In replating assay, MLL-AF9 was able to transform LSK, CMP, and GMP regardless of Hoxa9 status. Interestingly, gradually decreased in number of colony during RTTA was observed in MLL-AF9 transformed cells with Hoxa9$^{-/-}$ in all sorted populations when compared to wild type MLL-AF9 transformed cells (figure 4.41). These finding suggested that transformation efficacy of MLL-AF9 may suppress by genetic deletion of hoxa9.

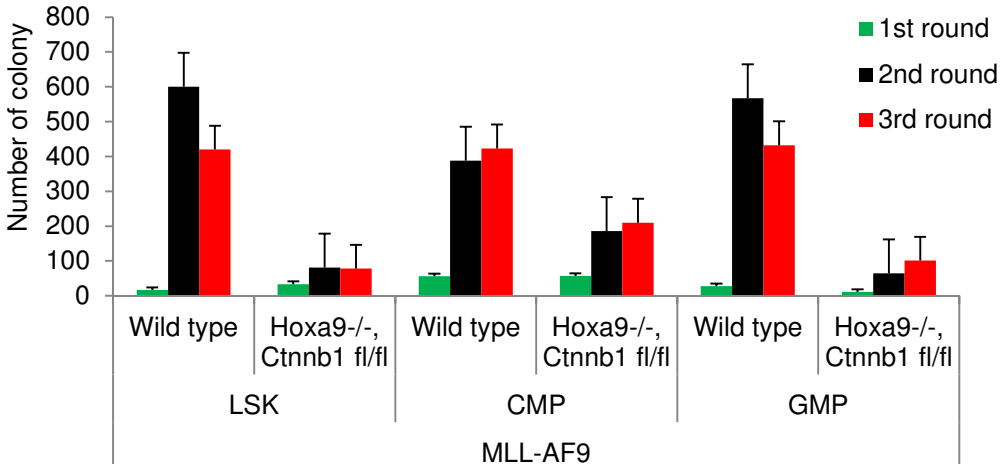


Figure 4.41: Knockout of Hoxa9 supress transformation efficacy of LSK-, CMP-, and GMP-MLL-AF9 in vitro. Absolute colonies number of LSK, CMP, and GMP cells isolated from bone marrow of wild type and Hoxa9$^{-/-}$/Ctnnb1^{flfl} mice transformed by MLL-AF9 during replating assay.

In addition to study the impact of single genetic deletion of β-catenin or Hoxa9 in the establishment of MLL-AF9 pre-LSCs derived from different cell of origins, I further investigated whether compound genetic deletion of β-catenin and Hoxa9 could abolish the transformation efficacy of MLL-AF9. Transformed LSK-, CMP- and GMP-MLL-AF9 cells with Hoxa9 knockout were conditionally deleted of β-catenin by administration of 20nM tamoxifen into the second round of RTTA followed by FACS sorting of YFP positive cells. As a result, genetic deletion of β-catenin and Hoxa9 could not suppress the transformation efficacy of MLL-AF9 pre-LSCs derived from LSK, CMP, and GMP in vitro (figure 4.42). To further investigated whether compound knockout of β-catenin and Hoxa9 could abolish the transformation efficacy of those pre-LSCs in vivo, LSK-and GMP-MLL-AF9 pre-LSCs with Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ and compound knockout of β-catenin and Hoxa9 were transplanted into recipient mice (n = 5 for each group).

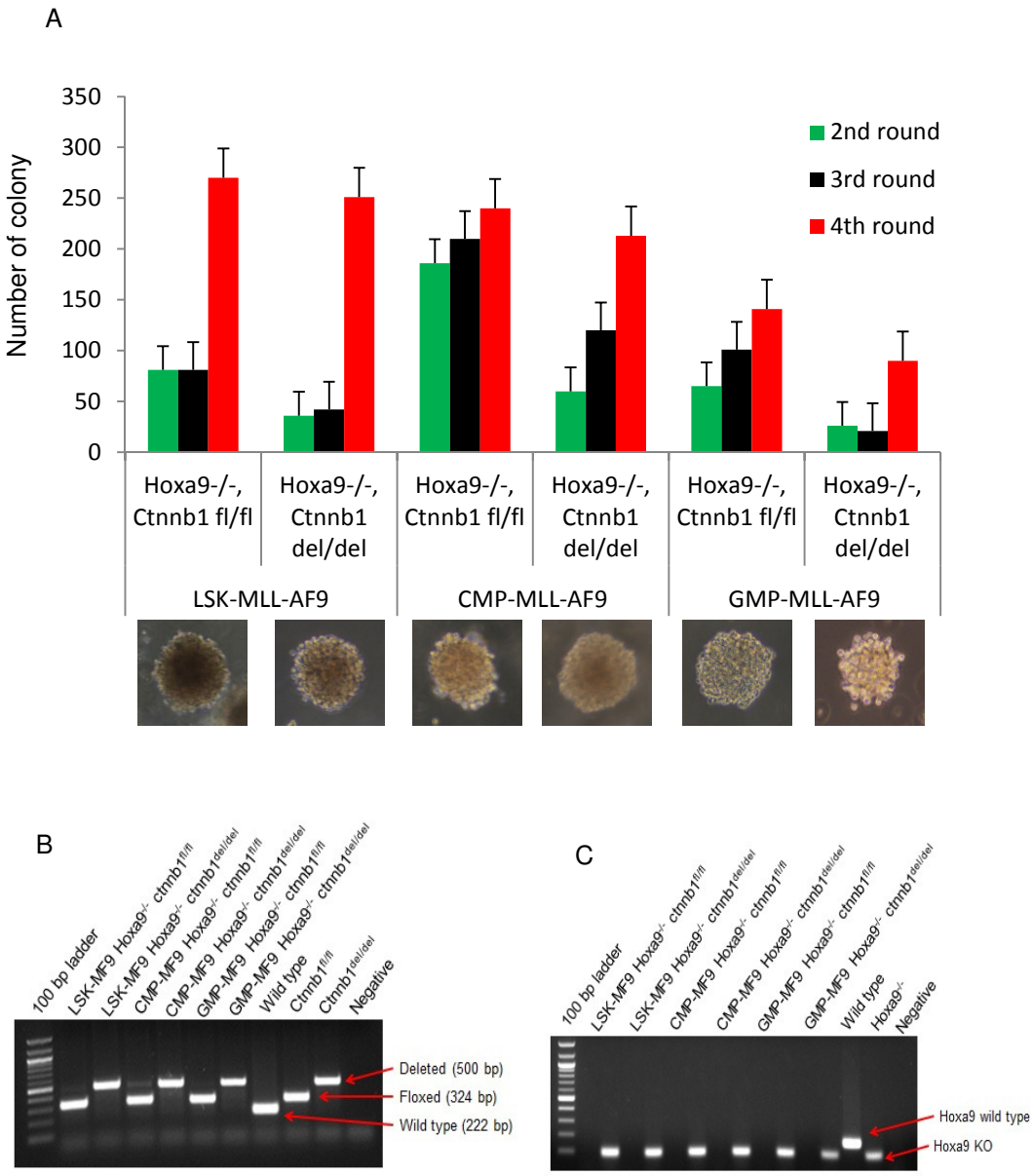


Figure 4.42: Establishment of LSK-, CMP, and GMP-MLL-AF9 pre-LSCs are β-catenin and Hoxa9 independent. A) Absolute colonies number and colony morphologies of LSK, CMP, and GMP cells transformed by MLL-AF9 during replating assay. B) and (C) Genotyping of Ctnnb1 and Hoxa9 on gDNA extracted from transformed cells after mediated deletion of Ctnnb1 gene by a treatment of 20 nM tamoxifen and YFP sorting.

Surprisingly, while mice that receive both pre-LSCs LSK- and GMP-MLL-AF9 with Hoxa9 knockout and Ctnnb1$^{fl/fl}$ cells was able to engraft and induce leukaemia (2 of 5 for LSK-MLL-AF9 and 4 of 5 from GMP-MLL-AF9), compound knockout of β-catenin and Hoxa9 abolished leukaemogenic property of both LSK and GMP derived MLL-AF9 LSCs in vivo (p = 0.1343 and 0.0133, respectively) (figure 4.43). Collectively, I could demonstrate the origin of

MLL-AF9 LSCs. Moreover, treatment of MLL-AF9 by targeting β-catenin and Hoxa9 may largely depend on cell of origin of MLL-AF9 LSCs. Furthermore, targeting multiple self-renewal pathways such as β-catenin and Hoxa9 which are dispensable for normal HSC but critical for LSCs could be a way of light to eradicate several types of AML LSCs.

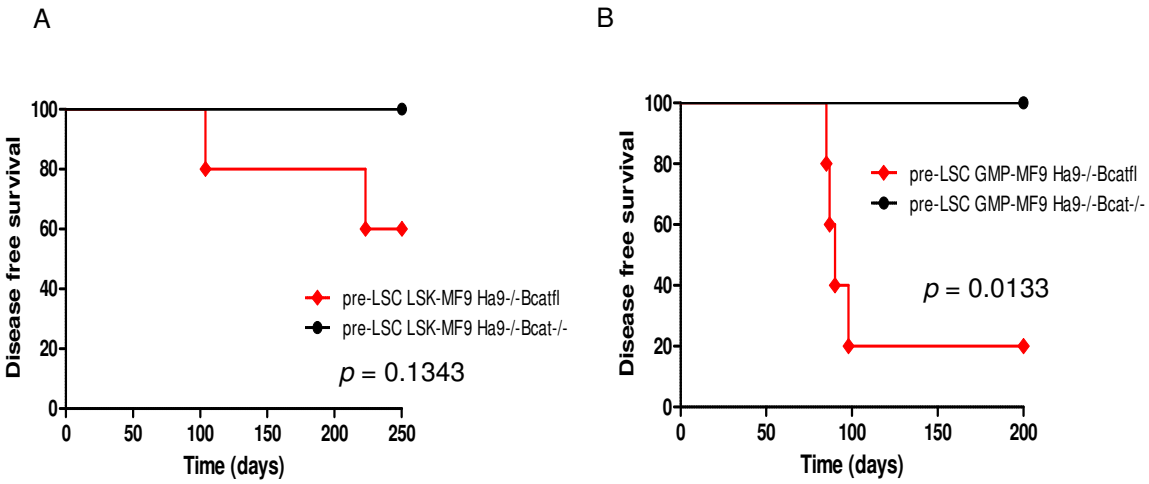


Figure 4.43: Combined genetic deletion of β-catenin and Hoxa9 abolish leukaemogenic potential of pre-LSC LSK-MLL-AF9 and GMP-MLL-AF9 in vivo. Survival curve of mice transplanted with pre-LSC LSK- (A) and GMP-MLL-AF9 (B) with Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ (n=5 for each group) and Hoxa9$^{-/-}$/Ctnnb1$^{del/del}$ (n=5 for each group).

Chapter 5 Discussion

Wnt/β-catenin has been characterized as an important molecule controlling normal and malignant haematopoiesis (Reya et al. 2003, Staal and Clevers 2005). Our group recently discovered that β-catenin is critical for transformation and mediating drug resistant properties of MLL LSCs derived from c-kit+ hematopoietic stem/progenitor cells (Yeung et al. 2010). It has also been shown that the expression level of β-catenin in different hematopoietic progenitors is essential for the initiation of LSCs by MLL-AF9 and Meis1-Hoxa9 (Wang Y 2010). However, it is unclear whether β-catenin also plays an important role in the development of LSC initiated from other recurrent LATFs derived from c-kit+ cells or functional distinct hematopoietic populations. In this report, I further investigated the impact of β-catenin in the initiation and maintenance of LSCs induced by common LATFs derived from both c-kit+ cells and distinct hematopoietic populations. Furthermore, I extended my work to the study of a novel functional crosstalk between Wnt/β-catenin and Hox pathways in the development of MLL LSCs derived from different cell of origins.

Using in vitro transformation assay (RTTA), conditional deletion of β-catenin did not impair the transformation efficacy of MN1, E2A-PBX1, and MLL-ENL pre-LSCs derived from c-kit+ hematopoietic stem/progenitor cells. Interestingly, while the development of E2A-PBX1 and MN1 LSCs were β-catenin independent, knockout of β-catenin abolished leukaemogenic property of c-Kit+ MLL-ENL LSC in vivo. These findings not only confirmed our group's previously published data that β-catenin is critical for the establishment of c-Kit+ MLL-ENL LSC (Yeung et al. 2010) using different mouse strain but also revealed the different functional requirements of β-catenin in the development of AML LSCs transformed by different LATFs. This finding also challenges the suggestion that β-catenin is generally required for development of AML stem cells (Wang et al., 2000).

MN1, a LATF not requiring β-catenin for development of LSC, is found not only as a fusion partner of chromosomal translocation t(12;22)(p13;q22) in several human myeloid leukaemia including AML, MDS, and CML but also over-expressed in human leukaemias (Grosveld 2007). Subsequent studies showed that MN1 is a very potent AML oncogene (Heuser et al. 2007). Here I showed that retroviral overexpression of MN1 in c-kit+ cells resulted in rapid induction of leukaemia in murine model regardless of β-catenin status. It has been documented that MN1 functions as a transcriptional co-activator cooperating with p300/CBP and RAC3 (van Wely et

al. 2003). In addition, high expression levels of MN1 correlated with observed ATRA resistance in AML patients (Heuser et al. 2007). Gene expression profiling of MN1 induced cell lines revealed that overexpression of MN1 resulted in the up-regulation of several leukaemogeneic oncogenes such as MEIS1 and BMI1 (Meester-Smoor et al. 2008). Remarkably, co-expression of Hoxa9 was able to enhance transformation efficiency of MN1-TEL leukaemia in mice (Kawagoe and Grosveld 2005). Thus c-kit+ MN1 LSC may compensate the absence of β-catenin with the up-regulation of other self-renewal pathways such as Hoxa9-Meis1 and BMI1 leading to initiation of MN1 LSC without β-catenin.

E2A-PBX1 is one of the most common fusion genes found in acute lymphoblastic leukaemia (ALL) and it has been shown that in murine models overexpression of several Hox genes such as Hoxa9 and Hoxb4 can accelerate the onset of disease (Bijl et al. 2008), suggesting a crosstalk between Hox and E2A-PBX1 in ALL. However, culture conditions in RTTA do not support lymphoid growth but are biased towards myeloid growth. Thus cells transformed by E2A-PBX1 in this assay have very different properties than E2A-PBX1 ALL cells and do not activate or require Hox genes for transformation (Cheung et al. 2007), suggesting that loss of β-catenin is compensated by Hox-Meis1 independent pathways during the development of E2A-PBX1 LSCs.

Interestingly, β-catenin has been recently reported to have a critical role in in another haematological malignancy, CML. Hu et al. 2009, demonstrated that purified BCR-ABL expressing LSCs (GFP^+ Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$) isolated from CML mice overexpressed β-catenin mRNA compared to normal HSCs (GFP^+ Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$) isolated from mice transplanted with empty vector transduced normal bone marrow cells. While BCR-ABL transformed BM cells with conditional deletion of β-catenin were able to home into the bone marrow of recipient mice, these cells failed to induce CML suggesting β-catenin is important in the initiation of BCR-ABL positive CML (Hu, 2009). In addition, Hidel et al. 2012, investigated the functional requirement of β-catenin for the maintenance of BCR-ABL induced CML. Conditional genetic deletion of β-catenin by tamoxifen in established CML (2 weeks or later after transplantation) did not result in a significant prolongation of the disease-free survival in mice transplanted with BCR-ABL transformed BM cells with Ers1-Cre Ctnnb1fl/fl genotype. However, in vivo β-catenin deletion led to a significant reduction in the number of CML cells in peripheral blood as well as in the total number of $Sca\text{-}1^+$ cells in bone marrow compared with tamoxifen treated control mice that received wild type BCR-ABL transformed BM cells. Interestingly, serial transplantation of CML

LSCs into secondary mice treated together with imatinib targeting BCR-ABL and tamoxifen to induce the deletion of β-catenin led to a reduction of Lin- and LSK fraction (enrich for LSC property) of CML cells in marrow. Strikingly, significant disease-free survival differences were only observed when these cells were serially transplanted into tertiary recipient mice, which has been phenocopied when Imatinib and the β-catenin inhibitor indomethacin had been used, suggesting that targeting β-catenin in combination with Imatinib may represent a novel therapeutic avenue in CML (Heidel FH 2012). Together, these findings reveal a different functional requirement of β-catenin in the establishment of LSCs depending on the leukaemia initiating events.

While MN1 and E2A-PBX1 were able to transform and establish AML LSCs in β-catenin deficient c-Kit+ cells which are a mixed and heterogeneous population of HSCs and progenitors, establishment of c-Kit+ MLL-ENL was β-catenin dependent. Similar to other MLL rearrangement leukaemia, MLL-ENL up-regulates several targets critical for the development of leukaemia. Zeisig et al, 2004 demonstrated that tamoxifen-inducible MLL-ENL-ERtm could transform and immortalise bone marrow haematopoietic cells when the culture medium was supplemented with tamoxifen (4-hydroxy-tamoxifen). Interestingly, gene expression analysis on those conditionally transformed cells revealed that Hoxa9, Hoxa7 as well as Hox coregulators meis1 and Pbx3 were up-regulated by MLL-ENL-ERtm. Moreover, while transformation efficacy of MLL-ENL-ERtm transformed cells were rendered by co-transduction with Bmi-1 (a known Hox repressor), single transduction with Hoxa9, Hoxa7, Meis1 or with both Hoxa7 and Meis1 only incompletely blocked differentiation of MLL-ENL-ERtm in the absence of tamoxifen. In contrast, MLL-ENL-ERtm cells co-transduced with both Hoxa9 and Meis1 resulted in sustained proliferation and arrested differentiation even when MLL-ENL-ERtm was inactivated (Zeisig BB 2004). Furthermore, Ayton and Cleary, 2003, demonstrated that MLL-ENL could not transform progenitor cells (Lin-) in the absent of Hoxa9 or Hoxa7 in vitro. In addition, while mice transplanted with wild type MLL-ENL transformed cells developed leukaemia, Lin- Hoxa9 knockout cells transduced with MLL-ENL did not induce leukaemia in recipient mouse. Moreover, co-transduction of Hoxa9 and MLL-ENL into hoxa9 deficient Lin- cells rescued both the in vitro clonogenic activity as well as the ability to induce leukaemia in recipient mice in vivo (Ayton PM 2003). These findings indicate that Hoxa9 and Meis1 are critical for transformation of MLL-ENL leukaemia derived from heterogeneous stem/progenitor cell populations such as c-kit+ or Lin- cells. Because c-kit+ cells are a mixed population of mostly non-self-renewing

progenitors (e.g., CMP and GMP) with a significantly fewer number of self-renewing HSC, I hypothesise that β-catenin dependent c-Kit+ MLL-ENL LSC may originate from populations that have limited self-renewal options/pathways. To test this hypothesis, I performed FACS sorting to purify HSC, CMP and GMP to investigate the origin of LSCs transformed by different LATFs as well as to test the different functional requirement of β-catenin in LSCs derived from different cell of origins.

To gain further insight into the cell of origins and role of β-catenin in transformation of different haematopoietic progenitors by different LATFs, HSCs, CMPs and GMPs from wild type and Ctnnb1$^{fl/fl}$ mice were sorted and subjected to RTTA and subsequently transplanted into recipient mice. In my preliminary experiments I showed that Meis1-Hoxa9 transformed and generated pre-LSCs in vitro originated from LSK, CMP, and GMP populations. In line with a recent publication (Wang Y 2010), I observed that LSK-Meis1-Hoxa9 pre-LSCs could induce leukaemia in mice and that deletion of β-catenin abolished the leukaemogenic property of LSK-Meis1-Hoxa9 pre-LSCs. Surprisingly, CMP-Meis1-Hoxa9 pre-LSCs also induced leukaemia in mice regardless of the β-catenin status, strongly suggesting a different requirement for β-catenin by the same oncoprotein in functional distinctive haematopoietic populations. Interestingly, although GMP-Meis1-Hoxa9 transformed cells could not develop leukaemia in recipient mice, constitutive overexpression of β-catenin in GMP-Meis1-Hoxa9 rescued the leukaemogenic capacity of those transformed cells. Gene expression analysis revealed that Cycloxygenase-1 (*Cox-1*) one of prostaglandin receptors (*Pstger1*) which is involved in the Wnt/β-catenin pathway was up-regulated in both LSK-Meis1-Hoxa9 and GMP-MLL-AF9 LSCs. Moreover, qRT-PCR and western blot specific to activated β-catenin revealed that β-catenin is overexpressed in both LSCs derived from LSK-Meis1-Hoxa9 and GMP-MLL-AF9. Interestingly, genetic deletion of β-catenin impaired the leukaemogenicity of GMP-MLL-AF9 transformed cells. These results suggested that β-catenin is critical for the establishment of LSK-Meis1-Hoxa9 and GMP-MLL-AF9 LSCs (Wang Y 2010). In contrast, my results, for the first time, reveal β-catenin independent transformation by Meis1/Hoxa9 in CMP population, suggesting that the cell of origin plays a critical role in determining the biology of the disease. To better understand the molecular mechanisms that contribute to β-catenin independence in CMP-Meis1-Hoxa9 LSCs, subsequent experiments including RNA sequencing on normal CMP versus CMP-Meis1-Hoxa9 Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ pre-LSCs are required.

On the other hand, I demonstrated that MN1 could only transform and establish LSCs in the CMP but not in the LSK or GMP population. Remarkably, similar to c-Kit+ MN1 LSC, initiation of CMP derived MN1 LSC was β-catenin independent. This finding was consistent with recent report that CMP is the cell of origin of MN1-induced leukaemia (Heuser et al. 2011). Interestingly, gene expression signature study revealed that normal CMP and MN1 leukaemic cells derived from unsorted bone marrow cells share a common gene expression signature including the specific up-regulation of *Meis1*, *Flt3*, and *Mef2c* compared with normal GMP. However, no significant differential expression of genes involved in Wnt/β-catenin in normal CMP and MN1 leukaemia cells was observed. Thus Wnt/β-catenin pathway may not be activated in transformed CMP-MN1 cells, which may at least in part explain its β-catenin independence. On the other hand, it has been reported that hoxa9 and hoxa10 are differentially overexpressed in MN1 LSC compared to mature myeloid cells, suggesting that initiation of MN1 LSC correlated with Meis1 and Hoxa9 expression (Heuser et al. 2006, Heuser et al. 2011). Consistently, similar to CMP-Meis1-Hoxa9, CMP-MN1 pre-LSC do not require β-catenin for the development of LSCs, suggesting that targeting of β-catenin may not be sufficient to eradicate LSCs with high Hoxa9 and Meis1 expression originated from common myeloid progenitor (CMP).

Together the data strongly demonstrate a different requirement for β-catenin in the development of AML LSCs originated from distinct hematopoietic populations and suggest that β-catenin independent LSCs may deregulate other self-renewal pathways such as Meis1/Hoxa9 in some cell populations to compensate for the loss of β-catenin.

MLL leukaemia is recognised as one of the most aggressive subgroups of haematological malignancies and confers very poor prognosis. It has been documented that MLL-AF9 transformed HSC as well as more differentiated GMP progenitors. Remarkably, leukaemogenic potential of GMP-MLL-AF9 was impaired by genetic deletion of β-catenin and pharmacologic targeting of β-catenin with indomethacin resulted in reduced numbers of GMP-MLL-AF9 LSCs (Wang Y 2010). However, it is not known whether HSC and CMP require β-catenin for the initiation of MLL-AF9 as well as other MLL fusion leukaemia. Using a different mouse strain, I confirmed the published result that loss of β-catenin impaired the leukaemogenic property of GMP-MLL-AF9. Surprisingly, I also found that HSC-enriched LSK- as well as CMP-MLL-AF9 induced leukaemia regardless of β-catenin status. To extend my work to other MLL leukaemias, I also studied MLL-ENL, which has been shown as an β-catenin dependent oncoprotein (Yeung

et al. 2010). In addition to demonstrating a similar β-catenin dependence for initiation of MLL leukaemia based on cell of origins, I also revealed an absolute dependence of β-catenin for the maintenance of the disease induced by LSC GMP-MLL-ENL. To further investigate the requirement of β-catenin in the maintenance of β-catenin independent, LSK-MLL-ENL, I have just recently repeated the experiment by increasing number of recipient mice as well as including LSC LSK-MLL-ENL controls. While additional experiments such as in vivo conditional deletion of β-catenin in MLL-ENL LSCs as well as treatment of MLL-ENL LSC transplanted mice with pharmacological β-catenin inhibitors are needed to assess the potential therapeutic targeting of β-catenin in different MLL-ENL LSCs, my data collectively provide strong evidence for different roles of β-catenin in the transformation mediated by the same fusion genes, depending on the cell of origins.

Intriguingly, I also demonstrated that different oncogenes/fusion genes display a different dependency of β-catenin. While MLL-ENL and MLL-AF9 require β-catenin for the transformation of more mature haematopoietic progenitors GMP, Meis1-Hoxa9 requires β-catenin for the development of LSC originated from HSCs-enriched LSKs. In contrast, MLL-ENL and MLL-AF9 transformed LSKs were β-catenin independent, suggesting LSCs originated from certain populations such as the HSC-enriched LSKs display differential β-catenin requirement dependent on the initiating events (e.g., Meis1-Hoxa9 vs MLL fusion). As *Meis1* and *Hoxa9* are critical genes upregulated by MLL fusion, these results would imply mechanistically, that other additional MLL target genes or deregulated pathways compensate for the loss of β-catenin in the development of LSK derived LSCs, and these pathways are absent in LSK-Meis1-Hoxa9 transformed cells. From a therapeutic point of view, the implications of these findings are twofold. While the oncogenic events influence the biology and therapeutic response of the disease, the origin of LSCs may also play an equal, if not more important role.

Self-renewal is a fundamental property and critical for both normal HSCs and LSCs. MLL, Hoxa9, Meis1, and β-catenin (Kroon et al. 1998, Dou et al. 2005, Sierra et al. 2006) are all recognized as critical molecules involved in normal HSC and LSC development. MLL increases Hoxa9, Meis1 and β-catenin expression levels (Balgobind et al. 2011). However, the expression levels of these proteins not only differ in distinct haematopoietic populations but are also influenced by the oncogenic events. In my studies, I demonstrated by q-RT-PCR that HSC-enriched LSK possessing self-renewal abilities highly express β-catenin, *Hoxa5*, *Hoxa7*, *Hoxa9*, *Hoxa10*, *Hoxa11*, *Hoxb4*, and *Meis1*, whereas down-regulation of those genes were observed

during differentiation from HSC to CMP and GMP progenitors. This observation is comparable to recent studies on the gene signatures of normal HSC/progenitors (Wang Y 2010). Interestingly, non-self-renewing GMP expressing low levels of β-catenin and Hoxa9 required both their presence to sustain leukaemic transformation by MLL-ENL and MLL-AF9. On the other hand, self-renewing HSC that abundantly express β-catenin, Meis1 and several Hox genes could still be fully transformed into LSCs by MLL fusions in the absence of β-catenin or Hoxa9. Several hypothese could be drawn from these results: Firstly, the inherent properties of the cell of origin determines if β-catenin is required for MLL LSCs. Secondly, MLL fusion target gene expression is influenced by the cell of origin resulting in β-catenin compensating pathways being activated by MLL fusion only in certain cell of origins resulting in β-catenin independent MLL LSCs. Thirdly, β-catenin may have different targets in different cell of origins resulting in variable outcomes of β-catenin deletion that may be only compensated by MLL fusion target gene expression in certain cell types. To address these hypotheses, I performed high throughput gene expression analysis on pre-LSCs LSK- and GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$ by using RNA sequencing technology. As expected, I could identify both origin-specific (selectively down-regulated in LSK or GMP in the absence of β-catenin) as well as origin-independent (common down-regulated in LSK and GMP in the absence of β-catenin) putative targets of β-catenin in MLL-ENL pre-LSCs. Interestingly, well defined, cancer-related β-catenin targets such as *c-myc* (He TC 1998), C*yclin D1*(Tetsu O 1999, Sansom OJ 2005), *Axin2* (Yan D 2001, Lustig B 2002, Jho EH 2002) were not affected in either LSK- or GMP-MLL-ENL pre-LSCs when β-catenin was deleted, suggesting that β-catenin targets in haematopoietic LSCs may be unique and different from other tissue-specific cancer stem cells. Moreover, knockout of β-catenin in pre-LSC GMP-MLL-ENL resulted in down-regulation of several genes involved in hypoxia and the control of myeloid cell differentiation. In contrast, these genes were not down-regulated in pre-LSC LSK MLL-ENL upon complete deletion of β-catenin. Consistently, Gene Set Enrichment Analysis revealed multiple pathways differentially expressed in pre-LSC LSK- and GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ and Ctnnb1$^{del/del}$. Together, these findings suggest that MLL pre-LSCs derived from different cell of origins have different β-catenin targets or deregulated pathways that are critical for the establishment of LSCs which in turn may determine whether MLL LSCs derived from different cellular origins are β-catenin dependent or independent.

In order to address whether β-catenin independent LSCs may utilize other self-renewal pathways such as Meis1/Hox to compensate for the loss of β-catenin, I determined the expression levels of several Hox genes and Meis1 in LSK- and GMP-MLL-ENL pre-LSCs. Hoxa9 was consistently found up-regulated in pre-LSC LSK-MLL-ENL compared to pre-LSC GMP-MLL-ENL using RNA sequencing as well as qRT-PCR,. Interestingly, while hoxa9 deficient progenitor cells (Lin- cells) can be fully transformed into LSCs by MLL-AF9 and MLL-GAS7 (So CW 2004), development of MLL-ENL LSC derived from Lin- cells was Hoxa9 dependent (Ayton PM 2003). Moreover, our group's unpublished data further revealed that GMP but not LSK required Hoxa9 for the development of MLL-ENL LSC. In addition, GSEA revealed that Meis1-Hoxa9 mediated pathway was consistently up-regulated in MLL-ENL transformed cell upon β-catenin inactivation. My findings suggest the presence of a functional crosstalk of β-catenin and Hoxa9 self-renewal pathway, which may operate to sustain β-catenin independent LSK-MLL-ENL LSCs.

To further investigate the functional crosstalk between β-catenin and Hoxa9 in the development of MLL-ENL LSCs, retrovirus carrying MLL-ENL and E2A-PBX1 were transduced into purified LSK, CMP, and GMP isolated from bone marrow of Rosa26-Cre/Rosa26-YFP/Hoxa9$^{-/-}$/Ctnnb1$^{fl/fl}$ mice and subjected to RTTA. While E2A-PBX1 was able to transform LSK, CMP, and GMP regardless of β-catenin and Hoxa9 status, MLL-ENL could not transform Hoxa9 deficient GMP cells, which is consistent with our group's unpublished data. More importantly, the transformation efficacy of LSK-MLL-ENL was impaired by combined genetic deletion of β-catenin and Hoxa9, while mice that received Hoxa9 deficient LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ developed leukemia, demonstrating a functional crosstalk between β-catenin and Hoxa9 in sustaining LSK-MLL-ENL LSCs. This result revealed that eradication of MLL LSCs may require the targeting of multiple self-renewal pathways. Interestingly, a critical p16/Arf suppression function of Hoxa9 in mediating Bmi1-independent LSC function has been recently proposed by our group (Smith et al. 2011), the functional relationship between β-catenin and hoxa9 in my work is less clear.

Globally RNA sequencing analysis on pre-LSC LSK-MLL-ENL with Hoxa9$^{-/-}$ and Ctnnb1$^{fl/fl}$ compared with LSK-MLL-ENL with Hoxa9$^{-/-}$ and Ctnnb1$^{del/del}$ revealed deregulation of several genes and pathways. Among those, genes involving in Meis1-Hoxa9 pathway including *Prmt1*, *Ruvbl1*, *Ruvbl2*, *Nolc1*, and *C1qbp* were down-regulated in the compound KO. Interestingly, *Prmt1* has been identified as a critical epigenetic component in certain MLL fusion

complexes critical for transcriptional regulation of hox genes (Cheung et al. 2007). A recent report demonstrated that *Prmt1* could methylate and stabilise Axin leading to increased levels of cytoplasmic β-catenin as well as enhanced β-catenin-dependent transcriptional activity, suggesting the presence of a positive feedback loop (Cha B 2011). However, whether molecular interactions between β-catenin, Hoxa9, and Prmt1 exist and how these molecules interplay with each other to facilitate the development of MLL-ENL LSCs remains unclear. Here, I hypothesise that Prmt1 could function as a key modulator in the development of MLL-ENL LSC in the absence of β-catenin or Hoxa9. Thus I performed shRNA-mediated knockdown of Prmt1 in LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ and Hoxa9$^{-/-}$ LSCs. Interestingly, knockdown of *Prmt1* resulted in the suppression of transformation efficacy of both LSC LSK-MLL-ENL Hoxa9$^{-/-}$ and LSC LSK-MLL-ENL Ctnnb1$^{del/del}$ in vitro. Consistently, while mice received LSK-MLL-ENL Hoxa9$^{-/-}$ transduced with empty vector control were able to develop leukaemia, knockdown of *Prmt1* abolished leukaemogenic potential of LSK-MLL-ENL Hoxa9$^{-/-}$ LSC, strongly suggesting that transformation efficacy of LSK-MLL-ENL LSC could be abolished by genetic deletion of Hoxa9 together with the suppression of *Prmt1*. Because LSC LSK-MLL-ENL with β-catenin knockout has a longer latency than LSK-MLL-ENL LSCs with Hoxa9$^{-/-}$, I am now waiting for the results of the remaining in vivo experiments using β-catenin knockout LSK-MLL-ENL LSCs. In addition to MLL-ENL LSCs, I extended my work and investigated the functional crosstalk between β-catenin and Hoxa9 also in the development of MLL-AF9 LSCs. Using a similar approach, I showed that MLL-AF9 transformed and established pre-LSCs of HSC, CMP, and GMP in vitro regardless of β-catenin and hoxa9 statuses. Interestingly, leukaemogenic potential of LSK- and GMP-MLL-AF9 pre-LSCs were abolished by genetic deletion of both β-catenin and Hoxa9, consistent with our finding on MLL-ENL. To exclude the possibility that the observed phenotype is solely due to the single loss of hoxa9, I have started control experiments in which LSK- and GMP-MLL-AF9 pre-LSCs with Hoxa9 knockout were transplanted into recipient mice (n=5 for each group) and I am waiting for in vivo data.

Together, my work provides the mechanistic foundation and rationale to target β-catenin and hoxa9 in HSC derived MLL fusion AML stem cells (figure 5.1). To translate these findings into the clinic and to achieve best possible treatment outcomes, patients should be stratified not only according to their genetic and cytogenetic features, but also their cell of origins. Although no robust strategy has been reported to predict on the cell of origin in individual AML patients, my results suggest this as one of the utmost important tasks for

designing better therapeutic regimes for cancer patients. Targeting multiple self-renewal pathways that are critical for LSC while dispensable or having a mild impact on normal HSC such as β-catenin and Hoxa9 could be a new therapeutic option to eradicate HSC derived MLL LSCs.

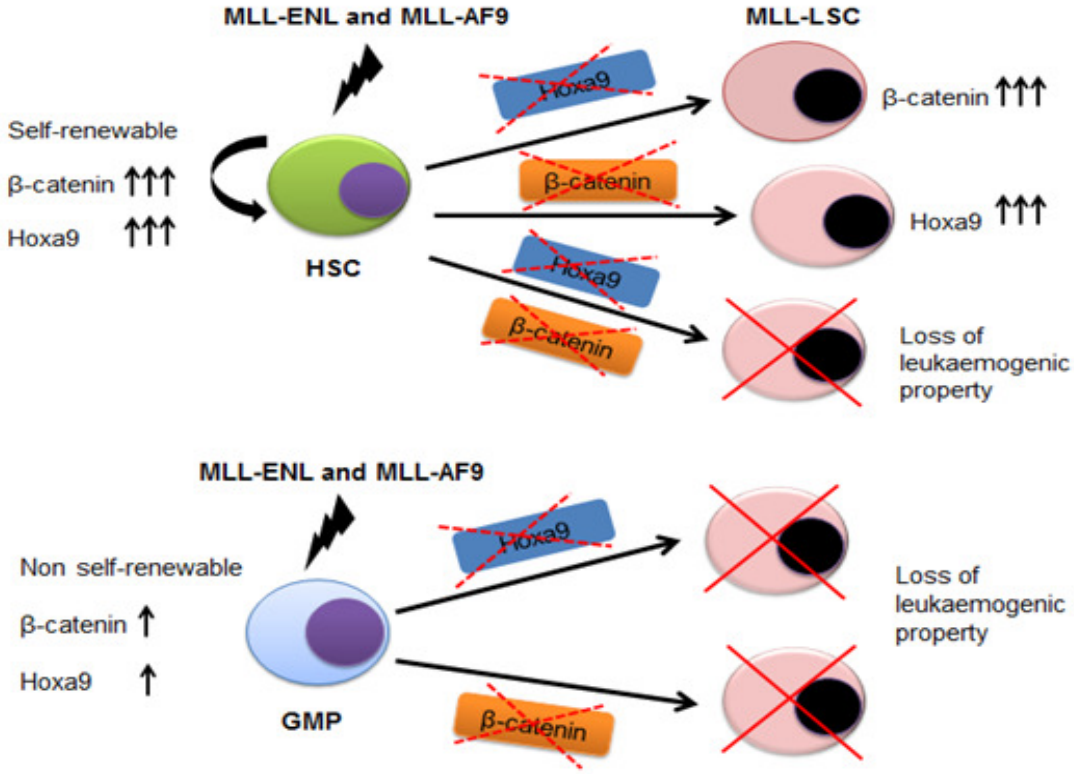


Figure 5.1: Diagram summarises the different in functional utilisation of β-catenin and Hoxa9 in the development of origin specific MLL LSCs.

Chapter 6 Conclusion

Molecular dissection of self-renewal pathways critical for normal HSC and LSC is very important to understand the biology of AML as well as to explore the effective therapeutic strategies. β-catenin is critical for the initiation of MLL LSCs, but is dispensable for normal HSC, highlighting it as a potential therapeutic target for selective eradication of LSCs while sparing normal HSCs. In this project, besides identification of the potential cell of origins of certain LATF transformed LSCs, I investigated the role of β-catenin in the initiation and maintenance of different LSCs derived from both c-Kit+ (mixed population of haematopoietic stem/progenitor cells) and functional distinct haematopoietic population (HSC, CMP, and GMP) using both RTTA (in vitro) and bone marrow transplantation assay (in vivo). LATF transformed LSCs derived from c-Kit+ cells showed different requirement for β-catenin in the establishment of AML LSC. Thus targeting of β-catenin largely depends on the initiating (LATF) events although it cannot be ruled out that also certain LATF transformed subpopulations within the c-kit+ compartment contribute to the observed β-catenin dependency. Although committed myeloid progenitor (CMP) thought to be the origin of β-catenin independent MN1 LSC, Meis1-Hoxa9 could transform and establish AML LSCs in LSK and CMP derived cells. Interestingly, while β-catenin was not required for establishment of CMP-Meis1-Hoxa9 LSC, knockout of β-catenin abolish leukaemogenic potential of LSK-Meis-Hoxa9 LSC. These findings further confirm the origin of MN1 LSC and indicate a different functional requirement for β-catenin in the development of LSCs induced by same LATF in different cellular origins.

MLL leukaemia is recognised as one of the most aggressive and poor prognostic subgroup of haematological malignancies. In this work, I observed that both MLL-AF9 and MLL-ENL transformed and established MLL LSCs in HSC, CMP, and GMP. Interestingly, while MLL LSCs derived from self-renewing HSC and CMP were β-catenin independent, the more mature progenitors, GMPs, required β-catenin for the development of GMP MLL LSC. These results strongly suggest functional differences of β-catenin in origin-specific LSCs in spite of their ability to induce phenotypically identical leukaemia. As β-catenin plays a crucial role to mediate stem cell self-renewal, I postulate the presence of multiple alternative self-renewal pathways in normal HSCs and certain progenitors allowing the transformation into origin specific LSCs in the absence of β-catenin. Analyzing gene expression data of normal LSK, CMP and GMP as well as transformed LSK-MLL-ENL and GMP-MLL-ENL revealed that Hoxa9 is overexpressed in

LSK and MLL-ENL transformed LSK compared to GMP and GMP-MLL-ENL transformed cells. In addition, our group's unpublished data revealed that Hoxa9 similar to β-catenin is critical for transformation of GMP-MLL-ENL whereas LSK-MLL-ENL LSCs are Hoxa9 independent. To further investigate whether Hoxa9 may functionally compensate loss of β-catenin for the initiation of MLL LSCs derived from LSK, I created a β-catenin/Hoxa9 knockout model where both genes can be inactivated in normal HSC and various myeloid progenitors. As a result, combined genetic deletion of β-catenin and Hoxa9 could abolish oncogenic potential of LSK MLL LSCs. Furthermore, Gene Set Enrichment Analysis (GSEA) on RNA sequencing data of pre-LSCs LSK-MLL-ENL with Hoxa9$^{-/-}$Ctnnb1$^{fl/fl}$ and Hoxa9$^{-/-}$Ctnnb1$^{del/del}$ revealed that several genes in Meis1-Hoxa9 pathway are down-regulated and which may contribute to the transformation into LSK-MLL-ENL pre-LSC. Among those, *Prmt1* is recognised as one of the critical components in certain MLL fusion complexes and crucial for transcriptional regulation of hox genes. Using shRNA knockdown to target *Prmt1* in LSK-MLL-ENL LSCs, I observed that *Prmt1* suppression impaired the oncogenic potential of LSK-MLL-ENL LSC in the absence of β-catenin or Hoxa9 in vitro. Consistently, Prmt1 depleted LSK-MLL-ENL Hoxa9$^{-/-}$LSCs could not induce leukaemia in recipient mouse, suggesting that Prmt1 may be a critical mediator for β-catenin function in LSK-MLL-ENL LSC.

Together, I have demonstrated, for the first time, specific molecular and functional differences among origin-specific LSCs, in which crosstalk between multiple self-renewal pathways such as β-catenin and Hox inherited from the cell of origins may result in resistance to current and future targeted therapies.

Appendix

Table A1: Gene differentially up-regulated in pre-LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Gene	Expression level (log2 fold change)
Asprv1	2.306517
Il18	1.995375
Serpinb2	1.958454
Trib1	1.763307
Serpinb10	1.661889
Col5a1	1.659591
Chi3l1	1.636033
Clec4a2	1.589804
Slc6a12	1.582576
B3galt5	1.546124
Plk2	1.539507
Pdlim4	1.51544
Fosb	1.502176
Camp	1.491799
Cd80	1.448872
Rhod	1.448068
Ipcef1	1.423693
Fos	1.418861
Pros1	1.383954
Plekhg1	1.381263
Olfm4	1.322393
Tmcc3	1.254155
Map6	1.253184
Lyz1	1.219912
Lyz2	1.139771
Bst1	1.133442
Hvcn1	1.133151
Mogat2	1.111229
Zfp819	1.047121
Clec4n	1.044438
Ces1d	1.018826
Ptafr	1.016371
Vcam1	1.005289

Table A2: Gene differentially down-regulated in pre-LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Samd5	-5.81334	*Nlrp3*	-1.61536
Ambp	-4.00272	*Adamtsl5*	-1.61304
Adm	-3.88804	*Vldlr*	-1.60235
Bpifc	-3.63531	*Lrrc32*	-1.59175
Myl10	-3.60336	*Htr2a*	-1.48824
Krt19	-3.43655	*4933439C10Rik*	-1.3937
Stc1	-3.22298	*Kcnc3*	-1.34549
Tgm2	-2.99229	*Lpar1*	-1.34092
Creb5	-2.88157	*Syn1*	-1.32871
F13a1	-2.58914	*Aldoc*	-1.31789
Irf4	-2.55722	*Htra3*	-1.20896
Tgfbr3	-2.53295	*Pgbd1*	-1.20839
Grin1	-2.44999	*Zkscan17*	-1.19713
Thbs1	-2.28755	*Maml3*	-1.19554
Fgf23	-2.22899	*Parp16*	-1.11956
Dusp4	-2.18131	*Dpep2*	-1.09721
Atp7b	-2.17041	*Kank1*	-1.07499
Mboat2	-2.14495	*Pafah1b3*	-1.06421
C3ar1	-2.05893	*Tex2*	-1.02854
Smtnl2	-2.05633		
Ccdc85a	-1.9999		
Mgarp	-1.99432		
Dlk1	-1.97149		
N4bp3	-1.96296		
St6gal1	-1.93236		
Nrbp2	-1.89797		
H2-Q10	-1.86999		
Ccr2	-1.84374		
Proser2	-1.77436		
Nckap5	-1.74207		
H2-Ab1	-1.69642		
G630025P09Rik	-1.67661		
Gm8773	-1.64746		
Rcor2	-1.62917		

Table A3: Gene differentially up-regulated in pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Ccl4	3.185838563	*Ms4a7*	2.045212866
Cxcl3	3.134265284	*Cd80*	2.044585525
Pla1a	2.965838519	*Lyz1*	2.044507986
Maf	2.855356589	*Il1f6*	2.04192448
Ccl2	2.849163441	*Il12b*	2.028364899
Clec4n	2.645477259	*Trib1*	2.026682829
Nrp1	2.582224497	*Ccl3*	1.993763386
Il1b	2.51805741	*Emr4*	1.993245226
Cxcl2	2.516556391	*Ccl9*	1.964412461
Astn2	2.515305902	*Ces2g*	1.952953296
Slc11a1	2.479411774	*Clec4a2*	1.943782942
Dst	2.463106384	*Plekhg1*	1.941066133
Cd302	2.442715198	*Ipcef1*	1.917239237
Tmc3	2.434509527	*Mmp13*	1.897428425
Cxcl1	2.428551123	*1100001G20Rik*	1.89479219
Csf3	2.394021565	*Tlr13*	1.890265777
1300002K09Rik	2.330119216	*Wfdc17*	1.888834575
Cd300e	2.325704503	*Ces1d*	1.888748555
C5ar1	2.307900969	*Aass*	1.882922653
Emr1	2.297342835	*Cd14*	1.870884623
Msr1	2.29494177	*Igf2bp1*	1.868112985
Orm1	2.266523148	*Mfsd6*	1.824232342
Slc7a11	2.239326023	*Wnt5b*	1.822569926
Cmklr1	2.224197601	*Stfa3*	1.821079503
Ms4a6d	2.21789825	*Acsl1*	1.820750249
Btla	2.212623315	*Plxna1*	1.811609783
Lox	2.201717482	*Dhrs9*	1.801665137
Cd9	2.199803314	*Mybpc3*	1.800914754
Il18	2.199471482	*Zc3h12c*	1.799309817
Pdpn	2.185685143	*Gja1*	1.798969127
Cd86	2.183804196	*Kctd12*	1.778566292
Plek	2.173610101	*Ccl6*	1.777767963
Fpr1	2.16287787	*Fstl1*	1.776612646
Lyz2	2.146090782	*Prdx1*	1.763221947
Mmp2	2.133453791	*Bst1*	1.748395792
Draxin	2.128094404	*Syt7*	1.748258198
Fcgr1	2.102112085	*Ppbp*	1.747674227
Marco	2.0912555	*Mmp9*	1.742736269
Vcam1	2.09009781	*Atp8b2*	1.723132243
Il1a	2.072542416	*6530402F18Rik*	1.71533675

Table A3: Continue.

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Scimp	1.709985418	*Met*	1.511626427
Mcoln2	1.704496217	*Bcl2l11*	1.510681754
Meis3	1.691405539	*Procr*	1.508639097
Irg1	1.685161639	*Gpr137b-ps*	1.506926565
Dgat2	1.681131588	*Gdpd1*	1.502388876
Cd200r4	1.678270373	*Slc6a12*	1.496163487
AF251705	1.672229715	*Dtx4*	1.494113686
Fpr2	1.664590053	*Dpp7*	1.49053851
Abca1	1.657061144	*Mrc1*	1.487825216
Pcdh7	1.644845931	*Hivep3*	1.48515487
Abca9	1.642201604	*Zmat3*	1.484243458
Slc7a2	1.635193838	*Adgb*	1.48327493
Il20rb	1.632322913	*Mgl2*	1.482911425
Heph	1.629385875	*Cfb*	1.478430139
Afp	1.627185459	*Sirpa*	1.474579363
Slc15a3	1.619106897	*Sgpl1*	1.464234927
Fmn1	1.61414124	*Ctsh*	1.464061223
Fcgr4	1.594106013	*Serf1*	1.463672408
Gbgt1	1.59348581	*Creb3l4*	1.454751513
Gm14023	1.592296058	*Abca8b*	1.452277117
Gm14047	1.588806611	*Csn2*	1.450664248
Clec4d	1.584564032	*Anxa1*	1.449400431
Cybb	1.581345985	*Phlda1*	1.449257236
Ift74	1.578540878	*Hsd11b1*	1.447202349
Saa3	1.573277737	*Clec4a1*	1.446979833
Slc39a14	1.573146133	*Rnf149*	1.446600223
Cd200r2	1.572753359	*Trim66*	1.439411244
4930515G01Rik	1.571856034	*Mpeg1*	1.435411694
Treml4	1.57106439	*Rbm24*	1.428644271
Tlr2	1.555798557	*Pla2g7*	1.427683089
Olfml2b	1.555024013	*Fam212b*	1.419331123
Dse	1.547201887	*Col5a1*	1.417745726
Olfm4	1.539790351	*Ptplb*	1.416049254
Col4a5	1.537739266	*Hspa2*	1.401949543
Ptges	1.536828824	*Fam196a*	1.398832829
Tnfaip6	1.536634652	*Cachd1*	1.393292809
Khdc3	1.533004596	*Il1r1*	1.392514538
Cd300ld	1.529065447	*Antxr1*	1.388535163
Trpm2	1.526288602	*Zfp385a*	1.388167943
Cd63	1.522454088	*41699*	1.38742872

Table A3: Continue.

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Marcks	1.382579938	*Lgmn*	1.302258517
Gpr137b	1.379243368	*Slfn1*	1.302056609
Serpine2	1.377097761	*Fth1*	1.301821065
Csf1	1.375840097	*Lrp1*	1.295290911
Rhoc	1.374635659	*Itih1*	1.291753964
Col4a2	1.372093442	*G630090E17Rik*	1.287604819
Selk	1.371315641	*Cd22*	1.283654484
Dcbld2	1.368945431	*Pdzd2*	1.282659441
Agpat4	1.368285895	*Acsbg1*	1.279848127
Ccl7	1.366810871	*Pilrb1*	1.277706332
P2rx6	1.366017578	*Wdr52*	1.27695564
Syne1	1.36410232	*Arhgef12*	1.267589847
Snord82	1.363780338	*Scamp5*	1.264453389
Plk2	1.363082676	*Tbc1d9*	1.263284325
Hpgds	1.357516509	*4930506M07Rik*	1.262494677
Trim29	1.354991834	*H2-M2*	1.258338422
Pdlim4	1.354840415	*P2ry6*	1.258047605
Spp1	1.351673797	*Havcr2*	1.256631619
Mdk	1.349427447	*Cd38*	1.255377945
1810011H11Rik	1.348050213	*Ms4a3*	1.25507721
Tmem104	1.346467885	*Htra1*	1.254694094
Fcrl1	1.342568887	*Gm5416*	1.253422405
Il10	1.34230399	*Gstt4*	1.252220267
Pld4	1.341688662	*9430069I07Rik*	1.252218966
Adora3	1.337658085	*Gpnmb*	1.250843765
Mir1983	1.336658362	*Snora75*	1.247973851
Dusp16	1.335241499	*Gbp2*	1.246757274
Stab1	1.331600386	*Gm16287*	1.245225824
Ms4a6c	1.330276607	*Hsph1*	1.244731408
Bud31	1.328123499	*Lamc1*	1.241335708
Siglece	1.324837276	*Cd36*	1.239175238
Cxcl5	1.323478176	*Fat1*	1.231713963
Bcl2a1b	1.323417403	*Serpinb2*	1.229312593
Snx7	1.317355994	*Pon2*	1.22797644
Il1rn	1.3164436	*Csf2rb2*	1.227439101
Cald1	1.312151327	*Tlr1*	1.226826255
Hdc	1.308174257	*Clec4e*	1.2266603
Hebp2	1.306997944	*D330045A20Rik*	1.224788172
Tmem132a	1.303772859	*Ntng2*	1.223094589
Csn1s1	1.303303674	*Ces2c*	1.220028003

Table A3: Continue.

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Tg	1.218765731	*Ggh*	1.159112843
Sgcb	1.216073648	*Lipf*	1.158839051
Fam167b	1.214554029	*Vcan*	1.154947934
Atp6v0d2	1.213189561	*Eng*	1.154281782
Pmp22	1.205786448	*Neto2*	1.152537856
Slc2a6	1.205617326	*Lipn*	1.151926367
Neo1	1.204075284	*Ndufa4*	1.149585389
Cxcl10	1.202443888	*Mmp8*	1.149382782
Snora33	1.201688495	*Atp2a2*	1.148430285
Sulf2	1.199776743	*Pira2*	1.148366957
N6amt2	1.198858653	*Kynu*	1.147105389
Cbr3	1.197119275	*Slc7a8*	1.145444153
Hist2h4	1.193110566	*Pf4*	1.145424603
E230025N22Rik	1.191548249	*Mgam*	1.14381944
Arg2	1.190529123	*Piwil2*	1.140827191
Ldhb	1.187994168	*Rab36*	1.139540155
Prune2	1.187922581	*Ube4a*	1.139433717
Gsta3	1.185863504	*Nabp1*	1.137557374
Abcg2	1.181862667	*Uros*	1.137248753
Trp53inp2	1.180044667	*Ccng1*	1.136870447
Slc25a18	1.178806703	*Ephx1*	1.136582496
Mrpl20	1.178551658	*Atp6v0b*	1.132071218
Pid1	1.178021211	*2010005H15Rik*	1.130884616
Cpeb1	1.176463944	*Tmem237*	1.130231357
Peg10	1.175916752	*Il6*	1.128196199
Slc39a2	1.175857302	*Fam134b*	1.126929691
Mmp14	1.175483523	*Slc35e4*	1.125479816
Plcb4	1.174088455	*Slc1a2*	1.124743427
Igsf3	1.171603251	*P2ry14*	1.123342593
Smim3	1.171423987	*Hist1h4h*	1.123148143
Armcx4	1.170867018	*Mmp27*	1.12257024
Slc6a8	1.170053453	*Tmem150b*	1.121896958
Fam219a	1.168373292	*Ifi47*	1.120412929
Ctsd	1.16554581	*Mgst1*	1.119607264
Snord12	1.163308839	*4732491K20Rik*	1.119452849
Mat2a	1.163128064	*5830432E09Rik*	1.116448216
Col5a3	1.163101803	*2310042D19Rik*	1.115697718
Tlr3	1.162960697	*Degs1*	1.115334418
Orm2	1.162734306	*Gusb*	1.114351435
Rnase4	1.160956987	*Vwf*	1.114158669

Table A3: Continue.

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Rab15	1.110289103	*Pde8b*	1.062097733
Ankrd22	1.109740442	*Fosl2*	1.061258025
2900060B14Rik	1.107931645	*Tmem251*	1.060216452
Tubb2a	1.107573177	*Lilra6*	1.059802229
Bcl2a1d	1.106523206	*Gm5531*	1.057627921
Bbs7	1.104550684	*Serpinb12*	1.056518318
Bax	1.100460768	*9530053A07Rik*	1.052030119
Srxn1	1.098217356	*Glyat*	1.051576607
Camp	1.09779431	*Rltpr*	1.050367853
Fndc9	1.097760349	*Glipr1*	1.049893131
Erp27	1.097015957	*Tomm5*	1.04945677
Avpr2	1.095505697	*Polr2l*	1.048791984
Dixdc1	1.094950515	*Serpinb9*	1.048678314
Gm5483	1.094608334	*Adam8*	1.048574613
Hvcn1	1.094316976	*Mfsd2a*	1.047634989
Fzd8	1.093937825	*Pno1*	1.04592583
Cpeb2	1.093624365	*Amh*	1.045381495
Ptger1	1.092255993	*Abcb9*	1.042339164
Kcnf1	1.090435907	*Tmem62*	1.041599529
Hist4h4	1.090098267	*Pqlc2*	1.040241743
Vnn3	1.089581397	*Plaur*	1.038863987
Ptprj	1.087331846	*Enpp2*	1.038481662
Naa20	1.086420487	*Pion*	1.037929643
Dennd5b	1.086182882	*Rbm44*	1.037763772
Rps27l	1.086110718	*Phlda3*	1.035023486
Pkib	1.084033458	*Akr1b8*	1.034079382
Slc31a2	1.083109519	*Cyp4a12a*	1.034029842
Fermt1	1.079318276	*Tmem216*	1.034015236
Gcnt2	1.075832091	*Tpcn2*	1.032787448
Pcdhgb7	1.07420027	*Tnni2*	1.032650587
Sall2	1.073912095	*Ear11*	1.032455144
Rnf128	1.07255144	*Ltf*	1.032174111
Tulp4	1.07092527	*Dclre1c*	1.03128709
Tal2	1.069767109	*Ppp1r16b*	1.029937436
Nlrp1b	1.068636273	*Rnu12*	1.028453689
Cyp4f18	1.06606946	*Plb1*	1.026827432
Fam181b	1.065905934	*Parp12*	1.026787402
Anapc10	1.065613276	*Lrrn4*	1.025150035
Eid3	1.063726042	*Pea15a*	1.023810279
Lphn2	1.062784101	*4933430I17Rik*	1.02045821

Table A3: Continue.

Gene	Expression level (log2 fold change)
Cacna1f	1.018466059
Nptn	1.018103964
Ccl25	1.016283947
Wdr4	1.015600932
Rab11fip1	1.015290741
Nmb	1.014912545
Ctsb	1.014557023
Tmem208	1.014539028
Map6	1.012912757
BC035044	1.012048254
2500004C02Rik	1.011975321
Gpr84	1.010140764
Reep3	1.008537849
Peli3	1.008054701
Fbxl22	1.007937566
Coprs	1.007053176
Bzw1	1.007036971
Hist1h2an	1.00690776
Gm11201	1.006213195
Clec4a3	1.005516233
Cd48	1.005308684
Tram2	1.005238198
Mrpl41	1.003262407
Cd68	1.002881058
Gm16023	1.001910704
Panx1	1.00085215
AU019990	1.000742871
Il13ra1	1.000699718
Proz	1.000509817

Table A4: Gene differentially down-regulated in pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Myl10	-3.28755	*Arhgdig*	-1.60209
Bpifc	-3.27585	*Repin1*	-1.59756
Irf4	-3.0374	*AI854703*	-1.59486
N4bp3	-2.98077	*Adrb2*	-1.57006
Tgm2	-2.46184	*Ccdc85a*	-1.53342
Nrbp2	-2.38744	*Lpar1*	-1.52918
Tgfbr3	-2.37423	*Dennd2a*	-1.52781
Tifab	-2.35449	*Adm*	-1.52096
Chpf	-2.10224	*Tsc22d3*	-1.49763
S1pr3	-2.06511	*Tgfb2*	-1.49719
Ambp	-2.05765	*Creb5*	-1.49271
Neurl1b	-2.03408	*Bzrap1*	-1.4893
F13a1	-2.0283	*Acss1*	-1.48811
Ptprf	-2.01235	*Gbe1*	-1.48476
Ifitm1	-1.9767	*Pou6f1*	-1.48339
Dlc1	-1.9388	*Sorcs2*	-1.47267
Zkscan17	-1.93655	*Klf9*	-1.46504
Mamdc2	-1.91642	*Grin1*	-1.46144
Pik3ip1	-1.91018	*Ccbl1*	-1.4585
Ndrg2	-1.87869	*Zfp57*	-1.44989
D630003M21Rik	-1.86034	*Kcnip3*	-1.44161
Elfn2	-1.83288	*Kif17*	-1.43356
Slc2a3	-1.80944	*Hdac7*	-1.42642
Il1r2	-1.80022	*Tmem65*	-1.42506
Vdr	-1.7966	*Iqgap3*	-1.41564
Maml3	-1.79133	*Mef2b*	-1.41476
Sbf2	-1.7794	*Rftn2*	-1.41455
Unc13a	-1.76948	*Echdc3*	-1.40427
St6gal1	-1.74666	*Dab2ip*	-1.40226
Zfp36l1	-1.74068	*Selenbp2*	-1.39214
Mxd1	-1.70461	*Bcr*	-1.38846
Selenbp1	-1.6779	*Haao*	-1.38839
Rhobtb1	-1.67771	*5031414D18Rik*	-1.38689
Zfp775	-1.66761	*Pycr1*	-1.38673
Zfr2	-1.65069	*Kank1*	-1.3755
4933439C10Rik	-1.64813	*Rnf208*	-1.36709
Smtnl2	-1.63921	*Fgf23*	-1.35241
Ddb2	-1.6299	*Dkkl1*	-1.34745
Slc27a2	-1.61735	*Cables1*	-1.34513
Gm10560	-1.61607	*Peli2*	-1.34247

Table A4: Continue.

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Rps6ka5	-1.33933	*Sdc1*	-1.21849
Reep6	-1.33114	*Klhl24*	-1.21552
Hspb6	-1.33106	*Arl5c*	-1.21393
Fbxo7	-1.32553	*Entpd3*	-1.21338
Rasa4	-1.32173	*Zfpm1*	-1.21206
Plekhh3	-1.31286	*Kctd19*	-1.20566
Tex2	-1.30957	*Rarg*	-1.20411
Hid1	-1.30264	*Tube1*	-1.20306
Tspan9	-1.29618	*Gm7694*	-1.20227
Lancl1	-1.29429	*Cask*	-1.20152
Ccdc138	-1.28949	*Spry2*	-1.19966
Pim1	-1.28509	*Proser2*	-1.19841
Samd5	-1.28391	*Adm2*	-1.18828
Elavl2	-1.27875	*Fbxl8*	-1.18721
2610015P09Rik	-1.27842	*Cspg5*	-1.1856
Extl1	-1.27736	*Slc9a5*	-1.1845
Dact3	-1.27635	*Als2cl*	-1.18391
Lrrc61	-1.27532	*Dmwd*	-1.18235
Dsp	-1.27266	*Etv4*	-1.17357
Plekhg3	-1.27111	*Cd244*	-1.17222
Nckap5	-1.26898	*Cbx4*	-1.1694
Mt2	-1.26875	*4933440M02Rik*	-1.16756
BC005764	-1.26398	*Isg20*	-1.16325
Tle6	-1.26283	*Ndrg1*	-1.16269
Angptl6	-1.25913	*Fbp1*	-1.15924
1600014C23Rik	-1.25771	*Lbh*	-1.15455
Prss16	-1.25762	*Fam64a*	-1.15361
H2-Q10	-1.25754	*4931417G12Rik*	-1.15304
Grik5	-1.25178	*Phf19*	-1.15169
AW011738	-1.25155	*Nupr1*	-1.15138
Fn1	-1.24973	*Samd10*	-1.14784
Npr1	-1.24371	*Nos1ap*	-1.14697
Zc3h6	-1.2407	*Muc13*	-1.14623
Matk	-1.23745	*Med12l*	-1.14478
Poli	-1.23517	*Cystm1*	-1.14259
LOC106740	-1.23355	*Hdac6*	-1.14171
Smad6	-1.23305	*E2f2*	-1.14122
Foxj1	-1.22526	*Epm2a*	-1.13654
Gm6297	-1.22471	*Slc52a3*	-1.1343
Tox	-1.21938	*Ptrf*	-1.13204

Table A4: Continue.

Gene	Expression level (log2 fold change)	Gene	Expression level (log2 fold change)
Map3k1	-1.13064	*Kcnq5*	-1.06419
Cdc25b	-1.13034	*Dpp4*	-1.0635
Adssl1	-1.12863	*Tfdp2*	-1.06325
Ptpru	-1.12572	*Ncapd3*	-1.06294
Pfkm	-1.12562	*Ttc25*	-1.06258
Thbs1	-1.11993	*Nat8l*	-1.0603
Kbtbd11	-1.11863	*Fancd2*	-1.05935
Stx11	-1.11696	*Rnf157*	-1.05933
Rasd1	-1.11615	*4921507P07Rik*	-1.05846
1700025G04Rik	-1.10942	*B3gnt8*	-1.05799
Fam83g	-1.1088	*Morn1*	-1.0578
Wnt9b	-1.10235	*Tmem51*	-1.05623
Atp7b	-1.10181	*Slc7a3*	-1.05613
Fgf11	-1.10082	*D930048N14Rik*	-1.05556
Crispld2	-1.09771	*Helb*	-1.05525
Wnt10b	-1.09716	*Gm4759*	-1.05468
Parp16	-1.0963	*Wdr65*	-1.05352
Cct6b	-1.09362	*Zfp618*	-1.0484
Gpr19	-1.093	*Cntnap1*	-1.04808
Ccno	-1.09185	*8430427H17Rik*	-1.04636
Gys1	-1.09126	*Llgl1*	-1.0458
Rad51b	-1.09005	*Adck3*	-1.04553
Pask	-1.08983	*Mmp28*	-1.04494
Dennd3	-1.08699	*Ldlrad4*	-1.04444
Cnbd2	-1.08615	*Dusp10*	-1.042
Gm1673	-1.08501	*Gpr56*	-1.04087
Klhl6	-1.08483	*Usp11*	-1.03887
Cep72	-1.08396	*Jazf1*	-1.03424
41888	-1.08341	*Gbp11*	-1.03123
Cgnl1	-1.08317	*Btbd2*	-1.0305
Tecpr2	-1.08273	*Aldoc*	-1.03002
Lsp1	-1.08049	*Me2*	-1.02937
Gpc3	-1.07705	*1500015A07Rik*	-1.02935
Hs3st6	-1.07612	*Dhx34*	-1.02591
Katnal1	-1.07606	*Rcor2*	-1.0257
Atp4a	-1.07391	*Wdr67*	-1.02424
Oas1d	-1.07224	*Ifitm5*	-1.02263
Kcnn4	-1.0715	*C920006O11Rik*	-1.01948
Fstl3	-1.06471	*Ncapd2*	-1.01926
Nfix	-1.06424	*Fam169b*	-1.01851

Table A4: Continue.

Gene	Expression level (log2 fold change)
Pold1	-1.01745
Pcsk4	-1.01443
Cenpf	-1.01431
Arrb1	-1.01371
Hif3a	-1.01302
Apobec3	-1.01274
Tjp3	-1.01256
Rnd3	-1.01068
D930028M14Rik	-1.01061
Prss57	-1.00954
Sbsn	-1.00935
Hist2h3c2	-1.00794
Hsf4	-1.00576
Suv420h2	-1.00562
Jak3	-1.00349
Lzts2	-1.00346
Pmaip1	-1.00204
Plekha6	-1.00096

Table A5: Gene specific up-regulated in pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Proz	*Tmem216*	*Fermt1*	*Fam134b*	*Slc39a2*
Il13ra1	*Cyp4a12a*	*Slc31a2*	*Il6*	*Peg10*
AU019990	*Akr1b8*	*Pkib*	*Tmem237*	*Cpeb1*
Panx1	*Phlda3*	*Rps27l*	*2010005H15Rik*	*Pid1*
Gm16023	*Rbm44*	*Dennd5b*	*Atp6v0b*	*Mrpl20*
Cd68	*Pion*	*Naa20*	*Ephx1*	*Slc25a18*
Mrpl41	*Enpp2*	*Ptprj*	*Ccng1*	*Trp53inp2*
Tram2	*Plaur*	*Vnn3*	*Uros*	*Abcg2*
Cd48	*Pqlc2*	*Hist4h4*	*Nabp1*	*Gsta3*
Clec4a3	*Tmem62*	*Kcnf1*	*Ube4a*	*Prune2*
Gm11201	*Abcb9*	*Ptger1*	*Rab36*	*Ldhb*
Hist1h2an	*Amh*	*Cpeb2*	*Piwil2*	*Arg2*
Bzw1	*Pno1*	*Fzd8*	*Mgam*	*E230025N22Rik*
Coprs	*Mfsd2a*	*Gm5483*	*Pf4*	*Hist2h4*
Fbxl22	*Adam8*	*Dixdc1*	*Slc7a8*	*Cbr3*
Peli3	*Serpinb9*	*Avpr2*	*Kynu*	*N6amt2*
Reep3	*Polr2l*	*Erp27*	*Pira2*	*Sulf2*
Gpr84	*Tomm5*	*Fndc9*	*Atp2a2*	*Snora33*
2500004C02Rik	*Glipr1*	*Srxn1*	*Mmp8*	*Cxcl10*
BC035044	*Rltpr*	*Bax*	*Ndufa4*	*Neo1*
Tmem208	*Glyat*	*Bbs7*	*Lipn*	*Slc2a6*
Ctsb	*9530053A07Rik*	*Bcl2a1d*	*Neto2*	*Pmp22*
Nmb	*Serpinb12*	*Tubb2a*	*Eng*	*Atp6v0d2*
Rab11fip1	*Gm5531*	*2900060B14Rik*	*Vcan*	*Fam167b*
Wdr4	*Lilra6*	*Ankrd22*	*Lipf*	*Sgcb*
Ccl25	*Tmem251*	*Rab15*	*Ggh*	*Tg*
Nptn	*Fosl2*	*Vwf*	*Rnase4*	*Ces2c*
Cacna1f	*Pde8b*	*Gusb*	*Orm2*	*Ntng2*
4933430I17Rik	*Lphn2*	*Degs1*	*Tlr3*	*D330045A20Rik*
Pea15a	*Eid3*	*2310042D19Rik*	*Col5a3*	*Clec4e*
Lrrn4	*Anapc10*	*5830432E09Rik*	*Mat2a*	*Tlr1*
Parp12	*Fam181b*	*4732491K20Rik*	*Snord12*	*Csf2rb2*
Plb1	*Cyp4f18*	*Mgst1*	*Ctsd*	*Pon2*
Rnu12	*Nlrp1b*	*Ifi47*	*Fam219a*	*Fat1*
Ppp1r16b	*Tal2*	*Tmem150b*	*Slc6a8*	*Cd36*
Dclre1c	*Tulp4*	*Mmp27*	*Armcx4*	*Lamc1*
Ltf	*Rnf128*	*Hist1h4h*	*Smim3*	*Hsph1*
Ear11	*Sall2*	*P2ry14*	*Igsf3*	*Gm16287*
Tnni2	*Pcdhgb7*	*Slc1a2*	*Plcb4*	*Gbp2*
Tpcn2	*Gcnt2*	*Slc35e4*	*Mmp14*	*Snora75*

Table A5: Continue.

Gpnmb	*Adora3*	*Anxa1*	*Gm14023*	*Aass*
9430069I07Rik	*Pld4*	*Csn2*	*Gbgt1*	*Wfdc17*
Gstt4	*Il10*	*Abca8b*	*Fcgr4*	*Tlr13*
Gm5416	*Fcrl1*	*Creb3l4*	*Fmn1*	*1100001G20Rik*
Htra1	*Tmem104*	*Serf1*	*Slc15a3*	*Mmp13*
Ms4a3	*1810011H11Rik*	*Ctsh*	*Afp*	*Ces2g*
Cd38	*Mdk*	*Sgpl1*	*Heph*	*Ccl9*
Havcr2	*Spp1*	*Sirpa*	*Il20rb*	*Emr4*
P2ry6	*Trim29*	*Cfb*	*Slc7a2*	*Ccl3*
H2-M2	*Hpgds*	*Mgl2*	*Abca9*	*Il12b*
4930506M07Rik	*Snord82*	*Adgb*	*Pcdh7*	*Il1f6*
Tbc1d9	*Syne1*	*Zmat3*	*Abca1*	*Ms4a7*
Scamp5	*P2rx6*	*Hivep3*	*Fpr2*	*Il1a*
Arhgef12	*Ccl7*	*Mrc1*	*AF251705*	*Marco*
Wdr52	*Agpat4*	*Dpp7*	*Cd200r4*	*Fcgr1*
Pilrb1	*Dcbld2*	*Dtx4*	*Dgat2*	*Draxin*
Acsbg1	*Selk*	*Gdpd1*	*Irg1*	*Mmp2*
Pdzd2	*Col4a2*	*Gpr137b-ps*	*Meis3*	*Fpr1*
Cd22	*Rhoc*	*Procr*	*Mcoln2*	*Plek*
G630090E17Rik	*Csf1*	*Bcl2l11*	*Scimp*	*Cd86*
Itih1	*Serpine2*	*Met*	*6530402F18Rik*	*Pdpn*
Lrp1	*Gpr137b*	*Cd63*	*Atp8b2*	*Cd9*
Fth1	*Marcks*	*Trpm2*	*Mmp9*	*Lox*
Slfn1	*41699*	*Cd300ld*	*Ppbp*	*Btla*
Lgmn	*Zfp385a*	*Khdc3*	*Syt7*	*Ms4a6d*
Csn1s1	*Antxr1*	*Tnfaip6*	*Prdx1*	*Cmklr1*
Tmem132a	*Il1r1*	*Ptges*	*Fstl1*	*Slc7a11*
Hebp2	*Cachd1*	*Col4a5*	*Ccl6*	*Orm1*
Hdc	*Fam196a*	*Dse*	*Kctd12*	*Msr1*
Cald1	*Hspa2*	*Olfml2b*	*Gja1*	*Emr1*
Il1rn	*Ptplb*	*Tlr2*	*Zc3h12c*	*C5ar1*
Snx7	*Fam212b*	*Treml4*	*Mybpc3*	*Cd300e*
Bcl2a1b	*Pla2g7*	*4930515G01Rik*	*Dhrs9*	*1300002K09Rik*
Cxcl5	*Rbm24*	*Cd200r2*	*Plxna1*	*Csf3*
Siglece	*Mpeg1*	*Slc39a14*	*Acsl1*	*Cxcl1*
Bud31	*Trim66*	*Saa3*	*Stfa3*	*Tmc3*
Ms4a6c	*Rnf149*	*Ift74*	*Wnt5b*	*Cd302*
Stab1	*Clec4a1*	*Cybb*	*Mfsd6*	*Dst*
Dusp16	*Hsd11b1*	*Clec4d*	*Igf2bp1*	*Slc11a1*
Mir1983	*Phlda1*	*Gm14047*	*Cd14*	*Astn2*
Cxcl2	*Il1b*	*Nrp1*	*Ccl2*	*Maf*
Pla1a	*Cxcl3*	*Ccl4*		

Table A6: Gene specific down-regulated in pre-LSC LSK-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Tifab	*Zfp57*	*Angptl6*	*Fam64a*	*Cgnl1*
Chpf	*Kcnip3*	*1600014C23Rik*	*4931417G12Rik*	*Tecpr2*
S1pr3	*Kif17*	*Prss16*	*Phf19*	*Lsp1*
Neurl1b	*Hdac7*	*Grik5*	*Nupr1*	*Gpc3*
Ptprf	*Tmem65*	*AW011738*	*Samd10*	*Hs3st6*
Ifitm1	*Iqgap3*	*Fn1*	*Nos1ap*	*Katnal1*
Dlc1	*Mef2b*	*Npr1*	*Muc13*	*Atp4a*
Mamdc2	*Rftn2*	*Zc3h6*	*Med12l*	*Oas1d*
Pik3ip1	*Echdc3*	*Matk*	*Cystm1*	*Kcnn4*
Ndrg2	*Dab2ip*	*Poli*	*Hdac6*	*Fstl3*
D630003M21Rik	*Selenbp2*	*LOC106740*	*E2f2*	*Nfix*
Elfn2	*Bcr*	*Smad6*	*Epm2a*	*Kcnq5*
Slc2a3	*Haao*	*Foxj1*	*Slc52a3*	*Dpp4*
Il1r2	*5031414D18Rik*	*Gm6297*	*Ptrf*	*Tfdp2*
Vdr	*Pycr1*	*Tox*	*Map3k1*	*Ncapd3*
Sbf2	*Rnf208*	*Sdc1*	*Cdc25b*	*Ttc25*
Unc13a	*Dkkl1*	*Klhl24*	*Adssl1*	*Nat8l*
Zfp36l1	*Cables1*	*Arl5c*	*Ptpru*	*Fancd2*
Mxd1	*Peli2*	*Entpd3*	*Pfkm*	*Rnf157*
Selenbp1	*Rps6ka5*	*Zfpm1*	*Kbtbd11*	*4921507P07Rik*
Rhobtb1	*Reep6*	*Kctd19*	*Stx11*	*B3gnt8*
Zfp775	*Hspb6*	*Rarg*	*Rasd1*	*Morn1*
Zfr2	*Fbxo7*	*Tube1*	*1700025G04Rik*	*Tmem51*
Ddb2	*Rasa4*	*Gm7694*	*Fam83g*	*Slc7a3*
Slc27a2	*Plekhh3*	*Cask*	*Wnt9b*	*D930048N14Rik*
Gm10560	*Hid1*	*Spry2*	*Fgf11*	*Helb*
Arhgdig	*Tspan9*	*Adm2*	*Crispld2*	*Gm4759*
Repin1	*Lancl1*	*Fbxl8*	*Wnt10b*	*Wdr65*
Al854703	*Ccdc138*	*Cspg5*	*Cct6b*	*Zfp618*
Adrb2	*Pim1*	*Slc9a5*	*Gpr19*	*Cntnap1*
Dennd2a	*Elavl2*	*Als2cl*	*Ccno*	*8430427H17Rik*
Tsc22d3	*2610015P09Rik*	*Dmwd*	*Gys1*	*Llgl1*
Tgfb2	*Extl1*	*Etv4*	*Rad51b*	*Adck3*
Bzrap1	*Dact3*	*Cd244*	*Pask*	*Mmp28*
Acss1	*Lrrc61*	*Cbx4*	*Dennd3*	*Ldlrad4*
Gbe1	*Dsp*	*4933440M02Rik*	*Cnbd2*	*Dusp10*
Pou6f1	*Plekhg3*	*Isg20*	*Gm1673*	*Gpr56*
Sorcs2	*Mt2*	*Ndrg1*	*Klhl6*	*Usp11*
Klf9	*BC005764*	*Fbp1*	*Cep72*	*Jazf1*
Ccbl1	*Tle6*	*Lbh*	*41888*	*Gbp11*

Table A6: Continue.

Btbd2	*C920006O11Rik*	*Arrb1*	*Prss57*	*Lzts2*
Me2	*Ncapd2*	*Hif3a*	*Sbsn*	*Pmaip1*
1500015A07Rik	*Fam169b*	*Apobec3*	*Hist2h3c2*	*Plekha6*
Dhx34	*Pold1*	*Tjp3*	*Hsf4*	*5430416N02Rik*
Wdr67	*Pcsk4*	*Rnd3*	*Suv420h2*	
Ifitm5	*Cenpf*	*D930028M14Rik*	*Jak3*	

Table A7: Gene specific differentially expressed in pre-LSC GMP-MLL-ENL with Ctnnb1$^{fl/fl}$ versus Ctnnb1$^{del/del}$ (p-value < 0.05 and fold change > 2.0).

Up-regulated gene (12 genes)		*Down-regulated gene (22 genes)*	
Mogat2	*Zfp819*	*Ccr2*	*Dlk1*
Serpinb10	*B3galt5*	*C3ar1*	*Gm8773*
Ptafr	*Rhod*	*Dpep2*	*Kcnc3*
Fos	*Tmcc3*	*Nlrp3*	*Pafah1b3*
Chi3l1	*Fosb*	*H2-Ab1*	*Mgarp*
Asprv1	*Pros1*	*Stc1*	*G630025P09Rik*
		Lrrc32	*Krt19*
		Htra3	*Adamtsl5*
		Dusp4	*Pgbd1*
		Mboat2	*Syn1*
		Vldlr	*Htr2a*

Table A8: Genes common differentially expressed in both pre-LSCs LSK- and GMP-MLL-ENL with in the absent of β-catenin (p-value < 0.05 and fold change > 2.0).

Common up-regulated gene (21 genes)		*Common down-regulated gene (31 genes)*	
Olfm4	*Hvcn1*	*Samd5*	*Rcor2*
Clec4a2	*Clec4n*	*Tgfbr3*	*Maml3*
Ces1d	*Pdlim4*	*Bpifc*	*F13a1*
Plekhg1	*Trib1*	*Irf4*	*H2-Q10*
Camp	*Ipcef1*	*Ambp*	*Kank1*
Lyz1		*Aldoc*	*Adm*
Lyz2		*Zkscan17*	*Smtnl2*
Plk2		*N4bp3*	*Parp16*
Cd80		*St6gal1*	*Proser2*
Col5a1		*Tgm2*	*4933439C10Rik*
Serpinb2		*Creb5*	*Thbs1*
Il18		*Nrbp2*	*Nckap5*
Slc6a12		*Myl10*	*Atp7b*
Vcam1		*Lpar1*	*Grin1*
Map6		*Tex2*	*Fgf23*
Bst1		*Ccdc85a*	

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